ANNUAL REPORT
OF
PROGRAM ACTIVITIES
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES
Fiscal Year 1980

U. S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service · National Institutes of Health

ANNUAL REPORT

OF

PROGRAM ACTIVITIES.

United States. NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES.

Fiscal Year 1980

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ANNUAL REPORT OF PROGRAM ACTIVITIES

NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

October 1, 1979 through September 30, 1980

Fiscal year 1980 continued the accelerated tempo of activity of the Office of the Director that has characterized recent years. While the Director stimulates much of this ever increasing pace, it stems even more from the rapid pace of scientific progress. A sense of excitement permeates the entire NIAID Directorate as a result of the outpouring of new information and technology resulting from past years of basic research. Recombinant DNA, hybridomas, suppressor T cells, helper T cells, Elisha and many other new words have had to be added to our vocabularies to accommodate these advances.

The Director continued a heavy schedule of invited lectures. During the fiscal year, the following lectures were given:

The Rufus Cole Lecture, "A Strategy for Prevention: The Study of Infectious Diseases from All Points of View," November 14, 1979. The Rockefeller University, New York, New York.

Introduction for Immunology of Human Infection, NIH, January 1980.

Seminars in Immunopathology, NIH, January 1980.

A Strategy for Prevention: "The Study of Infectious Diseases from All Points of View," January 29, 1980. Gorgas Memorial Institute, Washington, D.C.

A Strategy for Prevention: "The Beginning of Health Is To Know the Disease," April 4, 1980. Harvard, University, Boston, Massachusetts.

Dr. Robert Austrian: Recipient of the Philadelphia Award, April 9, 1980. The Barclay, Philadelphia, Pennsylvania.

A Common Goal. April 25, 1980. The Annual Meeting of the Asthma and Allergy Foundation of America, New York, New York.

Introductory Remarks: The Kinyoun Lecture. "The Molecular Cloning of E. Coli Enterotoxin Genes," May 5, 1980. Wilson Hall, NIH, Bethesda, Maryland.

Introductory Remarks: Sexual Harassment Seminar, May 8, 1980. Wilson Hall, NIH, Bethesda, Maryland.

Dedication Speech in Honor of Stephen Morse. International Symposium on Bacterial Vaccines, NIH, September 15, 1980.

Introductory Remarks, Human B. Cell Workshop, NIH, September 17, 1980.

Interferon Agenda, National Council of Drugs. Watergate Hotel, Washington, D.C., September 23, 1980.

Two highly significant studies were completed and reported to the NIAID Advisory Council, endorsed and made public. These were the reports on the Study Groups on Sexually Transmitted Diseases and on Immunology. These were intended to provide comprehensive state of the art reviews of the science and recommendations on the directions for future research. There was considerable constituent interest in the report on Sexually Transmitted Diseases, the American Social Hygiene Association arranging a wine and cheese reception in the House Office Building for Congressional Assistants to introduce the report.

Dr. Whitescarver attended a number of society meetings, contacting and discussing constituent thoughts about NIAID programs. Drs. Seal, Western, and Nutter also made similar contacts.

International Research

Dr. Karl Western joined the Office of the Director on October 1, 1979, as Assistant Director for International Research, replacing Dr. Joseph Cook. Dr. Howard Minners completed his assignment to the World Health Organization and transferred to the Office of International Health, HHS. The Director served on the Fogarty International Center (FIC) Task Force which completed its report and recommendations to the Director, NIH, then became a search committee for a new Director, FIC. He also served on the Scholars in Residence Selection Committee.

The Director went to Egypt in April with the Assistant Secretary for Health as a part of the U.S. Health Committee in its joint meeting with the Egyptian Committee. He also substituted for the Deputy Director as Chairman of the Subcommittees on Research and on Infectious Diseases that met during the committee meetings.

There was intensive consideration of NIAID interests and possible roles in collaboration with Chinese scientists. The Director is the coordinator for Immunology and corresponded with his Chinese counterpart to arrange the visit of a small American delegation to China in September for direct discussion of areas of mutual interest and possible collaboration. Traveling with the Director were Dr. Joseph Davie of St. Louis, Dr. Thomas Kindt of NIAID, and Dr. Gordon of CDC. Several high level Chinese visited the NIAID as part of visits to the NIH. Among them was Dr. Huang, Director of the Institute of Virology, Beijing. His visit offered opportunity to discuss the developing collaboration on viral hepatitis vaccine. The Minister of Health was briefed by the Deputy Director and Program Directors during his visit to the NIH and visited NIAID intramural laboratories.

There were many other foreign visitors for discussions related to mutual interests in the health problems of their countries.

Special Assistant to the Director

Dr. Jack E. Whitescarver, Special Assistant to the Director, has primary responsibility of serving as a focal point within the Institute for those professional and lay organizations that have an interest in the research programs of the NIAID. In this capacity, Dr. Whitescarver has attended several national meetings of constituent organizations. He assisted the Director and/or the Deputy Director in presenting fiscal and program data. On some occasions he spoke to the organization's Council or public affairs committees on the "public process" and presented Institutional information tailored to the host organizations. Meetings attended included the American Academy of Allergy, American Society for Microbiology, Cystic Fibrosis Foundation of America, and the National Reye's Syndrome Foundation.

On January 28, 1980, the same day the President's budget for FY '81 was announced, a "Program Review, FY '81" for the NIAID was held. Forty-seven organizations were invited and 45 sent representatives. Budget and program information was presented by the Director. Each Program Director cited plans for FY '81 that would be possible with the President's budget. Representatives from the constituent organizations engaged in discussion with the NIAID staff on various programs. The NIAID Profile FY '79 was distributed.

During the DRG Study Section meetings in June, Dr. Whitescarver arranged for several Congressional staff persons to attend. Those legislative aides who had previously expressed interest in NIH peer review were invited to observe. The Virology Study Section was selected for observation. Dr. Claire Winestock, the Executive Secretary, was very pleased to have the guests and conducted business as usual. Prior to the Study Section meeting, Dr. Whitescarver met with the Congressional staff and discussed the particulars in processing a grant application before the scheduled study section review. The event was well received by the aides and they each made positive comments on the NIH peer review process.

Office of Program Planning

During fiscal year 1980, this office continued to advise the OD on the development, analysis, and evaluation of the Institute's programs; provided support and liaison to the various program managers in coordinating, integrating and articulating long-range program goals and strategies; and served as the principal advisor on political and administrative developments which included projecting their impact on NIAID programs and operations. The major documents for which the OPP had lead responsibility were the Institute's Annual Research Plan and the material required for Congressional budget presentations. It also responded to requests from Trans-NIH Committees and other sources for program information and fiscal data.

During the fiscal year, Dr. Alfred M. Webb retired after having served for 14 years as chief of the office. Dr. Webb's long-term association with OPP had provided him with an unusual degree of insight into the programs of the Institute and their relationship to those of other Institutes and our scientific constituency. Although we will be able to continue to receive his advice as a reemployed annuitant (consultant), he will be sorely missed. During the year, Dr. Marilyn Bach joined the OPP by virtue of an IPA appointment and was assigned lead responsibility for developing the Institute's Evaluation Plan. Late in FY 1980, Dr. John E. Nutter was appointed Acting Chief of OPP. We are currently analyzing the functions of the office and considering how best to utilize the staff and expand their role to encompass the increasing requirement from NIH and DHHS for formal program evaluation efforts; the office was renamed the Office of Program Planning and Evaluation on August 1, 1980.

Office of Specialized Research and Facilities

The OSRF had primary responsibility during fiscal year 1980 for managing the NIH program to assess the risks of recombinant DNA research which involved several related efforts and component projects. The OSRF-supported projects that elicited the greatest interest from the Recombinant DNA Advisory Committee and members of the public were four contracts investigating various E. coli based host-vector systems and a workshop that focused on two potential risk scenarios.

Survival in the environment and the potential for selective advantage of organisms carrying recombinant DNA, should they survive, have been cited as matters of concern and NIAID had four contractors working on various aspects of this issue. The tests were performed in situations simulating accidental spills in the laboratory, in a model sewage treatment system, in mice and cultures simulating the mouse gastrointestinal system, and finally in germfree mice and humans. These projects provided direct data which supported the theoretical advantage of using disabled hosts and vectors in recombinant DNA research. In general, the E. coli K-12 systems survive less well in these natural environments than do "wild type" strains but, more importantly, a transfer of the vectors from certified hosts to the indigenous flora was searched for and not found.

The workshop was designed to define the scientific issues and assess the potential risks of: (a) possible direct adverse effects of hormone-producing strains of E. coli K-12; and (b) the possible occurrence of autoantibodies or autoreactive cells due to the production of eukaryotic polypeptides (including hormones) by E. coli K-12 should they colonize higher organisms. The purpose was to decide whether the two specific possibilities are valid scientific hypotheses if such an event did occur; and if valid, to determine if sufficient experimental data already exist to make a final judgment concerning possible risk. If data were insufficient, the participants were asked to outline those types of studies necessary to develop the definitive information on these issues. The meeting brought together 92 scientists from the fields of immunology, endocrinology, physiology, microbiology, infectious diseases and other appropriate disciplines. During the latter part of FY 1980, OSRF developed the Institute's response to two recommendations from the workshop to support appropriate studies to determine if concerns related to potential hazards of a breakdown of tolerance or adsorption of active peptides from the gastrointestinal tract have validity.

Part of the Risk Assessment Plan requires an annual update and OSRF has lead responsibility for construction of that document and the collection and collation of information from the NIAID Intramural Research Program, the other Institutes at the NIH, and various sources other than those directly supported by the Office itself.

During the year, a bilateral agreement was signed with the Japanese Government relating to nonenergy science and technology cooperation and one major component was recombinant DNA technology. The Director, NIAID, was designated as the U.S. governmental representative and OSRF has been interacting with all key individuals to organize the first meeting during FY 1981. It is anticipated that the initial meeting will focus on general topics of mutual interest and that speakers from both countries could address each issue from either the administrative/governmental or scientific viewpoint as appropriate. There was agreement that a substantial interchange could be achieved in relation to three topics: (1) guidelines for conduct or recombinant DNA work, (2) host-vector systems, and (3) risk assessment studies. By limiting the first meeting to these topics, a solid base would be established for future meetings which could address more specific issues.

The Recombinant DNA Guidelines mandate that each institution receiving NIH funds for such research have an Institutional Biosafety Committee. We will convene a meeting of the chairpersons of all U.S. committees in November, 1980; a previous meeting was held in November, 1978. The 1980 meeting will have, as one of its major purposes, the discussion of a proposed evaluation plan which will be implemented by a contractor and initiated near the end of FY 1981. The formal program evaluation is planned to acquire information helpful to both the institutions and the NIH in determining: (1) whether the IBC's as constituted can meet the increased responsibilities thrust upon them by the revision of the recombinant DNA guidelines and assure the protection of the laboratory worker, public and the environment; (2) solutions to institutional problems in the operation of IBC's and obtain information that will enable a more effective relationship between NIH and the institutions in the safe conduct of recombinant DNA research; (3) the relationships of IBC's to other institutional biohazard control and safety committees or mechanisms; and (4) needs for training courses and/or materials for IBC members and for entrants into recombinant DNA research.

OSRF has had, since its inception, the responsibility for high-containment facilities for the conduct of recombinant DNA research and other experiments should the need arise. During this fiscal year, several actions concerning these laboratories were taken. The Mobile Containment Laboratory and the facility under development in Building 41-T were transferred to the Division of Safety, NIH for their management in the future. The laboratories in Building 550 at the Frederick Cancer Research Center (FCRC) were recertified at the P4 level and, because of a lack of current requirements, the facility is being operated at lower levels of containment; it can be made operational as a maximum containment facility on very short notice should the need arise. The design of the National Biomedical Containment Laboratory (NBCL) considered for placement at the FCRC is now complete and the drawings restructured to permit a basic construction bid package with "add on's" to permit construction of the entire building in stages. Because of rapidly increasing construction costs and decreasing needs for such facilities, we do not recommend that the renovations of the FCRC buildings to establish the NBCL be initiated but that other approaches be investigated to satisfy NIAID and NIH needs for such facilities.

Office of Recombinant DNA Activities

Transfer of the Office of Recombinant DNA Activities (ORDA) from NIGMS to NIAID was completed at the beginning of FY 80.

The NIH Guidelines for Research Involving Recombinant DNA Molecules continued to evolve as major actions recommended by the Recombinant DNA Advisory Committee (RAC) were approved by the Director, NIH. The RAC met on December 6-7, 1979; March 6-7, 1980; June 5-6, 1980; and September 25-26, 1980. A major revision of the Guidelines was promulgated in the Federal Register on January 29, 1980. The revised Guidelines incorporate a new section, III-0, which specifies containment levels and administrative procedures for recombinant DNA experiments involving E. coli K-12 host-vector systems. Under Section III-0, most experiments involving E. coli K-12 can now be conducted under the lowest levels of physical and biological containment. In addition, these experiments no longer require the filing of a Memorandum of Understanding and Agreement with NIH. Experiments covered by Section III-0 need be registered only with the local Institutional Biosafety Committee (IBC). Saccharomyces cerevisiae host-vector systems were subsequently added to Section III-0 in July 1980.

The revised Guidelines also incorporate a new Part VI, entitled "Voluntary Compliance." This section stipulates that institutions, not otherwise covered by the Guidelines, may submit the membership of their IBC's to NIH for review and verification that they meet the requirements of the Guidelines. Part VI also states that institutions may voluntarily register with NIH information on recombinant DNA projects. This section also describes procedures that will be followed to protect proprietary information voluntarily submitted to NIH. During FY 80, more than a dozen institutions not otherwise covered by the Guidelines registered their IBC's with ORDA.

The Guidelines specify that the Director, NIH is responsible for authorizing, under procedures specified by the RAC, large-scale experiments (i.e., those involving more than 10 liters of culture) for recombinant DNA's that are rigorously characterized and free of harmful sequences. During FY 80, ten proposals to exceed the 10 liter limit were recommended for approval by the RAC and were approved by NIH, followed by a notice of such in the Federal Register. These ten proposals, all voluntary submissions from three companies, Eli Lilly and Company; Genentech, Inc.; and Burns-Biotec Laboratories, Inc., include attempts at large-scale production of human insulin, human growth hormone, and human leukocyte and fibroblast interferon.

On April 11, 1980, the NIH published in the Federal Register physical containment recommendations for large-scale uses of organisms containing recombinant DNA molecules. This document, while not formally part of the NIH Guidelines, is intended to serve as a model for those preparing large-scale submissions to the NIH under the Guidelines.

During FY 80, the RAC recommended and the Director, NIH approved the first exception to a prohibited experiment. The approval permitted the Plum Island Animal Disease Center to clone segments of the Foot-and-Mouth Disease virus for the purpose of developing a vaccine derived from a specific protein encoded by the virus. In July 1980, the Director, NIH approved a recommendation to permit removal of the clones from Plum Island to Genentech, Inc., which is collaborating with USDA in development of the vaccine.

ORDA continued to interact with other Federal agencies regarding recombinant DNA research and associated activities. The Director, ORDA, serves on the USDA Recombinant DNA Committee, which met on a frequent basis during FY 80. At the request of the Committee, ORDA arranged for evaluation of a USDA greenhouse located at Fort Detrick, Maryland, for its potential containment capabilities. The Director, ORDA, participated in a workshop of the Environmental Protection Agency (EPA) in San Diego, California, on November 16-17, 1979. The purpose of the workshop was to make recommendations for an EPA program to assess the potential impact of the introduction of new genomes into the environment. A solicitation for grant proposals based on the workshop subsequently was issued by EPA. The Director, ORDA, participated in meetings of the Federal Interagency Advisory Committee on Recombinant DNA Research during FY 80, and on June 4, 1980, participated in a workshop on insulins and growth hormone sponsored by the Food and Drug Administration.

ORDA participated in a briefing of Congressional staff members on the revised Guidelines on December 12, 1979, and the Director, ORDA, participated on March 18, 1980, in a meeting in San Francisco, California, sponsored by the Office of Technology Assessment. This meeting explored the impact of recombinant DNA technology on the pharmaceutical industry. The Director, ORDA, with senior NIH staff, participated in a hearing on May 20, 1980, on the industrial applications of recombinant DNA techniques before the Subcommittee on Science, Technology and Space of the Senate Committee on Commerce, Science and Transportation.

ORDA continued to interact with European policymakers during FY 80. On January 22-23, 1980, the Director, ORDA, participated, as the United States representative, in a meeting of the European Science Foundation Liaison Committee on Recombinant DNA Research. The role of the Committee is to foster common sets of standards for the conduct of recombinant DNA research among the countries of Europe and North America. The Director, ORDA, participated in a meeting with the Director, NIAID, and Japanese counterparts on February 13, 1980, on the recombinant DNA aspects of cooperation with the Japanese in nonenergy initiatives.

The Director, ORDA, serves as a consultant to the New York State Advisory Committee on Recombinant DNA, and participated in a meeting of that committee on June 9, 1980. This committee advises the State of New York on development of regulations to implement the State's recombinant DNA law.

The Director, ORDA, served as lecturer and discussion leader at seminars on Science, Technology and Public Policy sponsored by the U.S. Office of Personnel Management at Berkeley, California, on June 27, 1980, and at Oak Ridge, Tennessee, on September 4, 1980.

Office of Research Reporting and Public Response

ORRPR continues to get the "NIAID story" out in a variety of ways. Areas of program emphasis this year have included an expanded effort in audiovisual projects, extensive speechwriting and other editorial efforts for Dr. Krause, and greater distribution of NIAID publications.

We were fortunate in recruiting two senior technical publications writer-editors -- Nancy Brun and Phyllis Jay. Under contract, Lydia Woods Schindler assisted Dr. Krause in a variety of editorial assignments. She also edited the scientific monograph based on the Sexually Transmitted Diseases Study Group meeting in January. Reynard Biemiller, also under contract, advised ORRPR staff on layout and design of nonroutine publications. Tom Coleman completed his first year as an "expert in medical communications," primarily strengthening the Office's audiovisual capability. Mary Donovan left to become Assistant Chief of NIH's News Branch.

Publications -- The long-awaited lay version of the Asthma and Allergic Diseases Task Force Report, entitled "Asthma and Allergies -- An Optimistic Future," was completed and distributed to the nation's allergists, the Asthma and Allergy Foundation of America, the Institute's network of Allergy Research Centers, and selected medical writers. It is now on sale at the Government Printing Office. We plan to feature this book in an NIAID exhibit at the American Public Health Association meeting in October, 1980.

Another book for laymen, "Virology -- On the Edge of Life," designed in the format of Dr. Krause's "Director's Report" series, is in press and will be distributed during the Fall of 1980. To help save time in answering public inquiry requests, ORRPR staff produced two new publications: "Q & A About Genital Herpes" and "NIAID's Centers with an Allergy Research Component." Major revisions were completed on several fact sheets: "Hereditary Angioedema," "Sarcoidosis," "Acne," "Tuberculosis," "Q & A About Allergies," "Leprosy," and "Systemic Fungal Diseases."

Plans are under way to produce a handbook for the patient with lupus erythematosus. This publication will be a joint venture between the scientific and information staffs of NIAID and the National Institute of Arthritis, Diabetes, and Digestive Diseases.

Our staff also assisted in the production of (1) the administrative report, "Summary and Recommendations of the Sexually Transmitted Diseases Study Group," and (2) the scientific monograph incorporating the presentations and panel reports of the STD Study Group.

Distribution of NIAID publications for this year came to approximately 400,000. The sizeable increase over last year was due, in large part, to (1) working with GSA's Consumer Information Center, which helped to promote the publications in our allergy series, and (2) arranging for the staff of the Asthma and Allergy Foundation of America to assist us in distribution.

Public Inquiries -- ORRPR staff prepared approximately 700 individualized letters in answer to public inquiries and answered about 3,000 telephone inquiries from the public. An estimated 80 letters were answered under the Freedom of Information Act procedures.

Congressional Reports -- We prepared two special reports on Sexually Transmitted Diseases for the House and Senate Appropriations subcommittees. In addition, we prepared NIAID sections to five special reports to the Congress on genetic diseases, cystic fibrosis, diabetes, digestive diseases and arthritis.

Scientific Meetings and Information-- One staff member managed the publicity of the NIAID Consensus Development Conference on the Use of Amantadine for Influenza. This included arranging a press briefing and disseminating the post-conference report.

Exhibits were produced and manned by ORRPR staff at the annual meetings of the following constituent organizations: The American Academy of Allergy, Atlanta; the American Society of Microbiology, Miami; the American Lung Association, Washington; the Lupus Foundation of America, Knoxville; and the "Clinical Meetings" (American Federation for Clinical Research, the American Society for Clinical Investigation, and the Association of American Physicians), also in Washington. Other exhibits were produced for meetings of the Group on Public Relations of the Association of American Medical Colleges (on the NIH campus) and the Philippine Medical Association (Washington). All of these exhibits had a videotaped message and a display of appropriate NIAID publications. ORRPR staff also assisted the Institute's EEO Coordinator in preparing exhibits.

Our staff also worked closely with Dr. Gordon Wallace in developing publicity for NIAID's Kinyoun Lectures, established last year by Dr. Krause to emphasize the interdependence of infection and immunity. This year's lecturers were: Dr. Purnell Choppin, Rockefeller University; Dr. Rupert Billingham, University of Texas, Dallas; Dr. Dorothy Horstmann, Yale; and Dr. Stanley Falkow, University of Washington.

Three articles were written by ORRPR staff for the NIAID section of the Journal of the American Medical Association. Topics covered were vasculitis, antiviral substances, and occupational asthma.

Patient Education -- ORRPR is in the process of completing its second slide/tape show, "How to Cope with Your Allergy -- At Home, At School, and On the Job." The first slide/tape show, "What You Should Know About Asthma," has been offered for sale by GSA's National Audiovisual Center. Nearly 400 programs (consisting of 40 slides, an audiotape, and a printed script) have been sold to physicians, schools, voluntary health organizations, and hospitals.

Films -- ORRPR has planned the production of feature films on genital herpes, lupus and on NIAID's Intramural Research Program. A cinematographer from NIH's Audiovisual Branch has been assigned to do the camera work. Filming has been scheduled at intramural laboratories in Bethesda and Montana, as well as at grantee institutions where genital herpes research is being conducted. We also are working with NIH's Audiovisual Branch to develop radio and television public service announcements for national distribution (2,500 radio stations and 750 television stations). An announcement on the use of flu and pneumonia vaccines is slated for the 1980-81 season.

Library -- Reference sources in ORRPR's library continue to expand to provide up-to-date scientific information for Institute personnel. The library now receives about 90 journals, with 10 journals added this year. Sixty texts have also been added to the library this year.

Administration

The major personnel change during the year was the transfer of Ms. Marianne Wagner, Personnel Officer, to the National Cancer Institute, and her replacement by Ms. Janie Lacy who came from NCI.

A very large workload was imposed in efforts to implement the Personnel Reform Act. The Director, Deputy Director, Executive Officer, all members of the Senior Executive Service, and those who come under the Merit Pay Plan attended one or more days of formal training courses. The Director was appointed to serve as Chairman of one of the NIH Performance Review Boards. The Deputy Director held multiple meetings with SES members reporting to the Director's office, discussing objectives and performance with the Director and with the staff member. He also met several times with managers under the Merit Pay Plan that are in the Office of the Director and supervised by him. The Management Analyst was given added duties to gather and disseminate information, maintain records, and to work with the Executive Officer and Deputy Director in implementation of the Act.

Still another addition to the workload that required much of the Executive Officer's time was implementation of the current "1 for 2" personnel freeze. This has required a constant stream of discussions and reports to try to maintain ability to meet critical needs while reducing staff through attrition.

On a whole, and despite the ever increasing workload created by travel, personnel, parking, and other restrictions or reporting requirements, administrative activities have been efficiently performed. The NIAID takes pride in its staff. Mr. Robert Steiner, Administrative Officer, RML, and Ms. Jo Morris, Travel Assistant, OD, received NIH Merit Awards for their work.

Equal Employment Opportunity

The major events relate to the intramural research program. Most significant was the great success of the Introduction to Biomedical Research meeting held at the National 4-H Center in February. This meeting, arranged by Dr. Kay Cook Jaouni, brought in graduate or medical students from the MBS program. In addition, it found new sources in other colleges or universities resulting in Native Alaskans and American Indians, more Hispanics, and a very representative cross section sample of minorities on racial and sexual bases. Many of these students and others were given temporary appointments in intramural laboratories during the summer. Some are in the Co-Step program. This pool will be nurtured and followed with the hope of eventual gains from it to the staff of highly qualified minority scientists and physicians.

The EEO Advisory Committee Charter was revised and submitted to the Director. The biggest change was in election of members to assure that all organizational units were represented.

The EEO Coordinator attended a number of meetings such as the National Medical Society, MBS Program, National Urban League, where he directed exhibits, distributed literature on the NIH, its programs and opportunities, and discussed possible future employment with those that had questions.

Dr. Theresa Mercado and Ms. Ena Camargo were active on the Hispanic Committee, arranging for and presenting an excellent program to BID Directors and the Office of the Director, NIH.

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Immunology, Allergic and Immunologic Diseases Program
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REPORT OF THE DIRECTOR

IMMUNOLOGY, ALLERGIC AND IMMUNOLOGIC DISEASES PROGRAM

Fiscal Year 1980

A. Administrative Summary

1. Organization and Function

The Immunology, Allergic and Immunologic Diseases Program (IAIDP) was initiated on October 1, 1976, coincident with the reorganization of NIAID into major programs characterized by professional function. With this change the IAIDP assumed the responsibility for research, training, and conference activities that formerly were assigned to the Allergy and Immunology Branch of the Extramural Programs and contract activities in transplantation immunology and immunologic research resources previously administered within the Collaborative Research Program.

During FY 1980, the IAID Program operated according to the followed organizational plan:

Office of the Director

Director-Sheldon G. Cohen, M.D.
Special Assistant to the Director-Dorothy D. Sogn, M.D.
Assistant for Program Management-George R. Jenkins, B.S.
Assistant for Scientific Review-Justina S. Grauman, M.S.

Clinical Applications Section (CAS)
Head-Daniel I. Mullally, M.D., M.P.H.

Allergy and Clinical Immunology Branch (ACIB)

Chief-Robert A. Goldstein, M.D., Ph.D.
Special Assistant-Thomas T. Hubscher, Ph.D.

Immunology and Immunochemistry Branch (IIB)

Chief-Bernard W. Janicki, Ph.D.
Program Officer-John G.Ray, Jr., Ph.D.

Genetics and Transplantation Biology Branch (GTBB)

Chief-Henry Krakauer, M.D., Ph.D.
Serum Bank Manager-Katherine A. Hopkins, R.N.

Each of the three IAIDP Branches assumes responsibility for administering investigator initiated research grants and collaborative research contract awards, developing programmatic initiatives and new projects, serving on trans-NIH coordinating committees and taking active roles in the development and conduct of workshops and conferences in areas of the biomedical and biological disciplines and the clinical medical specialties with which they are concerned.

All three Branches are also involved with programs related to Research Career Development Awards and training, i.e., individual and institutional fellowships. In addition, the Allergy and Clinical Immunology Branch administers Allergic Disease Academic Awards which are designed to further the careers of mid-level investigators on career tracks in Academic and Research Allergy and the Young Investigator Research Grant Awards designed to assist and encourage investigators in early stages of their careers to develop research interests and capabilities in immunology, especially relevant to clinical aspects.

The IAID Program also assists investigators by procuring and providing certain reference standard and research reagents not otherwise available from commercial sources. These have included human and mouse histocompatibility typing sera, purified allergens, and antisera to lymphocyte subsets. In keeping with Program staff's responsibility for maintaining adequate inventories of reagents as well as for developing new sources and identifying new materials for acquisition, this year a new endeavor was initiated. By contract with the American Type Culture Collection a mechanism has been developed which will permit IAIDP to systematically and rationally acquire, store, characterize and distribute cell lines produced by the fusion of spontaneously proliferating and differentiated cells, e.g., myeloma and antibody - producing lymphocytes. These, monoclonal antibody-producing, cell line (hybridomus) available to the concerned research communities, as well as to contractors charged with providing this Program and its serum bank with reagents.

During FY 1980, IAIDP/NIAID negotiated and awarded a contract to the Mt. Sinai Medical Center, Milwaukee, Wisconsin for the maintenance of an International Bone Marrow Transplantation Registry, relevant to the goals of the Genetics and Transplant Biology Branch of the IAID Program. The data to result from this project is expected to contribute more information on the immune processes and immunological problems associated with bone marrow transplantation. This Registry represents a unique resource to the bone marrow transplantation community.

The IAIDP/NIAID Clinical Research Centers Programs are now comprised of 17 Asthma and Allergic Disease Centers (AADC) and 4 Centers for Interdisciplinary Research on Immunologic Diseases (CIRID). A new venture undertaken during FY 1980 resulted in expansion in scope of the AADC program to include special efforts directed to immunologically mediated skin diseases. Two Immunodermatology Centers established at an approximate total first-year support of $500,000 are located at New York University and at the University of Texas Southwestern Medical College in Dallas. Interdisciplinary approaches incorporated in both clinical and basic scientific studies were designed to investigate immunologic mechanisms which may play a role in the pathogenesis of certain cutaneous diseases. The Centers will utilize the combined efforts of clinicians, immunologists and cell biologists. Cellular and humoral immunologic, biochemical, ultrastructural and cell culture techniques will be employed to investigate the pathogenesis of allergic contact dermatitis, lichenoid skin diseases, cutaneous vasculitis, lupus erythematosus, the blistering diseases (pemphigus, bullous pemphigoid) scleroderma, dermatomyositis, the porphyrias, mycosis fungoides and mast cell mediated inflammation. These studies are expected to provide a clearer understanding of the basic mechanisms involved in the pathogenesis of cutaneous disorders and aid in the development of new therapeutic approaches.

During FY 1980, by a three year contact awarded to the Sidney Farber Cancer Institute an investigation will be conducted on immunosuppressant effects of a series of agents including corticosteroids, cytotoxic drugs, antilymphocyte/thymocyte serum/globulin and total lymphoid irradiation. The current practice of immunosuppression in human organ transplantation, in diseases of the immune system, and in cancer chemotherapy is highly empirical. In the first two instances, the objective is from reaction against the patients own or against the engrafted tissues. Under such circumstances, the use of immunosuppressants can be counter-productive either because, when too profound, it entails too great a risk of susceptibility to infection, or because it may so disturb the regulation of the immune system as to bring the rejection process totally out of control. Consequently, the definition of the effects of immunosuppressants on lymphocyte subpopulations with defined functions (helper, supressor, effector) will be of paramount importance in permitting the rational, tailored utilization of these agents.

The first steps in a new endeavor have been taken by issuance of a Request for Research Grant Applications (RFA) on the genetic and structural characterization of minor non MHC and tissue-specific alloantigens. This project is designed to stimulate research in a difficult and underserved area of potentially great significance to transplantation. To date, determinations of histocompatibility have focused entirely on the products of genes comprising the major histocompatibility complex (MHC) as antigens on lymphocytes. It has become clear, however, that matching of donors and recipients of grafts at the MHC loci has limited predictive power for the success of the transplantation. This may be due in some cases to disturbances of immunoregulation induced by immunosuppressive regimens resulting in a form of autoimmunity or vigorous reaction to normally weak antigens, or, probably more frequently, to the presence of polymorphic antigens coded by loci other than MHC or specific for the transplantation tissue.

"The Clinical Trial of Skin Testing with Major and Minor Penicillin Derivatives in Hospitalized Adults" is now underway. Reagents were distributed to each of 8 participating centers in June of this year. Centers including UCLA, University of Washington at Seattle, Northwestern, University of Colorado, Rochester, Cornell, Walter Reed Army Medical Center, and the Clinical Center at NIAID are engaged in investigating whether skin testing with major and minor determinants of penicillin can predict immediate hypersensitivity reactions within one hour of administration of penicillin (or its analogues) in history positive and in history negative hospitalized adults. If this predictive value is shown, the study may further demonstrate whether a positive or negative history adds information in this respect, beyond that gleaned from skin tests. It is hoped that approximately 5,000 patients will be enrolled in the study over a two year period.

An Immunology Study Group was organized in November 1979 and is comprised of 98 members working in six panels dealing with regulatory mechanisms, hybridoma technology, cloning, molecular genetic approaches to immunologic systems, cell membrane antigens and effector mechanisms. This group is focusing on the state of immunologic knowledge in those areas where important developments are most likely to occur and to identify areas where special support is needed. In turn, it is anticipated that priorities among those determinations will be assigned. Publication of a full report is anticipated in early 1981. The panel reports and recommendations, now in draft stage, will be considered by a subcommittee of the NIAID National Advisory Council in September in preparation for presentation to the full Council later in the fall. The Council summary of that report and recommendation for funding in each of these six important areas will be published in a smaller edition some time in 1981.

The rapid developments and progress in research on physiologic function, chemistry and genetics of immunocompetent cells have not only been important for the fundamental knowledge revealed but additionally have pointed to areas where clinical relevance may be. Accordingly, this year we have instituted efforts to facilitate interactions and exchanges between the Directors of Program Projects in Lymphocyte Biology.

Our mission as the designated lead Institute in the study of immune mechanisms has alerted us to the work of other NIH B/I/Ds concerned with organ and disease oriented interests involving dysfunctions of the immune system. Accordingly, we have developed joint program efforts especially related to conference activities and definition of research initiatives with NIAMDD (dermatology, diabetes), NEI (occular immunopathology), NCI (tumor immunology) and NHLBI (hypersensitivity lung diseases).

The CAS has been pursuing leads suggested by a clinical observation that asthma in children residing in certain inner city areas has been noted to have assumed a pattern of increasing severity and frequency. With the collaboration of the MIDP Epidemiology and Biometry Branch the initial phase of a study is currently in process utilizing the medical record facilities at the Children's Hospital National Medical Center of DC. In this institution the diagnosis of asthma has been verified as the leading cause of admissions among children not under continuing outpatient care, increasing ten fold over the past 25 years. Attempts will be made to evaluate possible relationships to identifiable socioeconomic, environmental and etiologic - pathogenic factors to explain this phenomenon. Plans are also being drawn up to investigate whether this may be only a local phenomenon or may represent a national trend.

The work of a study group on organ specific alloantigens was directed to a review of evidence that tissue specific antigens, independent of the MHC antigens, have demonstrable actions in graft rejections. Existing and new technologies available for characterization of tissue antigens have been considered. The findings and opinions are expected to stimulate additional investigative and evaluatory work in an important but neglected area.

The data collection and correction of the Kidney Transplant Histocompatibility Study (KTHS) has been completed and the first stage of evaluation and analysis effected. This work consisted of characterization of donor and recipient populations, of selection, operative and postoperative procedures, of outcomes, and of certain elementary correlations among them. Emerging from this analysis is verification that transfusions have a beneficial effect but confound many other variables such as HLA matching at AB loci so that to evaluate any other procedure, careful adjustment must be made for the transfusion history of the recipient. Splenectomy has a beneficial effect. Age and race but not sex of the recipient have significant impact in cadaveric transplants. In living related donor-recipient transplants, serologic matching at A and B loci are important but even more so is the reaction of recipient lymphocytes to donor cells in the MLC test. The utility of antithymocyte globulin again has been demonstrated to be equivocal with the interpretation of data complicated by variations among participating centers. This survey has had the objective of furnishing leads to the world community for extended studies both based on the data already available from the study and for additional studies that are in process by the GTTB designed to permit firm conclusions to be drawn.

Results of this multi center observational study have been documented in a NIAID publication available from the Government Printing Office. This 2 volume publication gives detailed tabular material and comprehensively describes donors and recipients, the practice of transplantation and the result achieved at participating hospital centers. The second phase of study involving extended data analysis has been initiated through collaboration with the Harvard School of Public Health and the Peter Bent Brigham Hospital.

Research Resources Program

The major histocompatibility complex (MHC) of the mouse continues to be a focal point of research interest in experimental immunogenetics because of the effect of this series of closely linked genes on tissue transplantation, susceptibility to viral infection and cell interactions during immune responses. The IIB appreciating the biomedical significance of these studies inherent in the similarity of the mouse H-2 complex to the MHCs of higher vertebrates including man has been responsible for providing important reagent material to researchers working in this area of NIAID programmatic interest and responsibility.

During the past year through the contract mechanism, it has been possible to supply newer immunologic H-2 complex reagents to grantees, contractors, and other qualified investigators seeking to understand and possibly manipulate immune mechanisms by antigen recognition control and immunotherapy. Newer specificities of H-2 antisera related to tissue histocompatibility and specific Ia antisera involved in immune response mechanisms have been identified. Ly antisera specific for thymus-dependent lymphocytes applicable to studies of cell sub-sets in immune mechanisms and other Ly antisera specific for nonhistocompatibility cell marker studies, tumor specific antibodies and theta cell antisera have been developed, banked and distributed.

Initiation of a Hybridoma Cell Bank (discussed in an earlier section) represents an innovative nationwide cooperative endeavor under the leadership of IAIDP/NIAID.

Serum Bank Activities

The policy pertaining to contracts for the acquisition of homozygous typing cells is in the process of re-evaluation. Data from reference laboratories evaluating sera offered to the Bank are now analyzed. In-house studies seeking to evaluate reproducibility, scoring and technique variations between laboratories have been undertaken. The "NIH technique" or assay has been redescribed in an attempt to standardize its use and to recommend a more accurate scoring method, the objective being to achieve greater reproducibility and comparability among laboratories.

Typing sera trays have been redesigned for the purpose of developing a basic clinical tray and an extended tray to meet research needs. These are in the process of production, the plan being for ultimate preparation and distribution of the basic tray to be placed in the hands of a private organization to meet clinical practice needs. It is anticipated that this step will serve to effectively initiate transition of resources developed by NIAID to commercial suppliers. Since more recent approaches to DR serum typing are indicative of promising advancements, a DR locus typing tray is being designed. Laboratories working in this new area have been identified as sources of appropriate serum sample contributions.

A data acquisition and analysis capability is being developed in the serum bank for the purpose of conducting in-house studies on pooled national data in the area of immunogenetics.

The inventory and ordering procedures for bank sera and the evaluation of reference laboratory reports has now been completely automated. Confidence in the Serum Bank by the transplantation immunology community continues to grow as evidenced by some 700 sera offers, one-third of which represented rare specificities, since the new bank policies were instituted.

Publications

The GTBB has prepared and initiated issuance of a new catalogue supplement for the users of histocompatibility testing reagents.

The detailed report of the five-year kidney transplantation histocompatibility study under the supervision of the GTBB including statistical tables has been completed and published through the Government Printing Office (GPO).

Summaries of these investigations from the standpoint of the statistical analysis and findings and results at collaborating centers have been prepared by NIAID advisory panel members and consultants and are in the process of publication.

Barnes, B.A., and Oliver, D.: Transplantation Proceedings. (in press)
Jonasson, O., and Simmonds, R.L.: Transplantation. (in press)

Proceedings of the GTBB Conference on "Organ Specific Alloantigens" has been prepared and is scheduled for publication Transplantation Proceedings in a forthcoming special supplementary issue.

A catalogue of homozygous typing cells that can be made available as research resource reagents to qualified research scientists is in the process of assembly by the GTBB.

The manual of tissue typing techniques used in immunologic research has undergone revision and a new addition distributed to approximately 750 research investigators, institutes and medical libraries.

The lay version of the report of the Task Force on Asthma and the Other Allergic Diseases entitled "Asthma and Allergies, an Optimistic Future," was completed, received from the GPO in July 1980 and immediately placed in the distribution process.

Based upon published material in the NIAID report of the Task Force on Asthma and the Other Allergic Diseases, the IAIDP ACIB is working with the American Academy of Allergy Scientific and Educational Council in the development of manuscript material for a "Primer in Allergy."

A project has been initiated as a cooperative effort with NIAMDD to develop a "Handbook for the Lupus (SLE) Patient."

Proceedings of the Workshop on "Basic and Clinical Aspects of Granulomatous Diseases" are scheduled for publication in book form (Elseiver-North Holland). A comprehensive summary for prompt distribution prepared by Institute staff is in the process of publication at the GPO.

2. Budget

The following shows the distribution of support by award mechanism for the activities of the Program during FY 1980.

Immunology, Allergic and Immunologic Diseases Program
FY 1980 Awards

Award Mechanism		Number	Amount*
Research Grants		446	$ 48,361,688
Career and Career Development Awards		47	1,757,966
	Subtotal	493	$ 50,119,654
Fellowship Awards		52	622,273
Training Programs		32	2,353,311
	Subtotal	84	$ 2,975,584
Contracts		37	1,959,226
	Total	614	$ 55,054,464

*Total costs, indirect costs estimated

Conference Support

During FY 1980, the following meetings, conferences, and workshops relevant to the activities and functions of the Immunology, Allergic and Immunologic Diseases Programs were conducted by the IAIDP.

November 1979	Asthma, Allergic and Immunologic Diseases Centers Workshop, Bethesda, MD
December 1979	First National Workshop on Organ Specific Alloantigens, New York, NY
June 1980	Workshop on Basic and Clinical Aspects of Granulomatous Diseases, Bethesda, MD (jointly with the Division of Lung Diseases, NHLBDI)

As an integral part of a program based upon a directive from the U.S. Senate Appropriations Committee to develop a cordinated effort to further progress on immunologically mediated insults to the eye, IAIDP/NIAID staff collaborated with the National Eye Institute in the design and conduct of a series of three workshops based on introducing interactions between immunologic and vision research scientists.

December 1979	Immunogenetics and Transplantation Immunity
March 1980	Autoimmune Phenomona and Ocular Disorders
June 1980	Infection, Inflammation and Allergy

Additionally IAIDP programatic support was given to the following national and international conference and workshop endeavors.

February 1980	Gordon Conference on Immunochemistry and Immunobiology, Santa Barbara, CA
March 1980	Conference on Control of Cellular Division and Differentiation, Keystone, CO
April 1980	Monoclonal Antibodies: A Perspective for the Future, St. Louis, MO
	Working Group Meeting on Total Lymphoid Irradiation in Organ Transplantation and Autoimmune Disease, Stanford,CA
June 1980	8th International Congress of the Transplantation Society, Boston, MA
July 1980	Fourth International Congress of Immunology, Paris, France
August 1980	2nd International Congress for Cell Biology, Berlin, West Germany

B. Scientific Summary

The Immunology, Allergic and Immunologic Diseases Program (IAIDP) is concerned with immune system functions in health and disease. Programmatic activities are oriented to cellular and humoral antibody mediated aspects of immune phenomena, pathogenic aspects of immune disorders and allergic inflammation and disease, and the genetic relationship of immune responsiveness to transplantion biology. Within these programmatic activities focus is placed upon original work directed to studies on the structure, function and basic biology of the immune system and applications to pathophysiologic changes. Responsibility for involvement in these biomedical areas is accomplished through investigator initiated research projects, collaborative research and development contracts, clinical investigative centers concerned with asthma, allergic and immunologic diseases, lymphocyte biology and immunologic diseases program projects, and the procurement and distribution of research resources and reference reagent materials including the maintenance of a serum bank for histocompatability testing materials.

Designed to accomplish these objectives, the IAIDP is organized into three biomedical and scientific branches dealing with:

1. Immunobiology and Immunochemistry
2. Allergy and Clinical Immunology
3. Genetics and Transplantation biology

Through the work of these three programmatic functional entities, IAIDP maintains insight and remains alert to important developments and aspects of both basic and clinical endeavors in immunology and allergy. Progress in immunobiology, immunochemistry and immunogenetics is continually under review and awareness maintained in a continuous search to exploit potential applications of basic research data to problems concerned with health and disease involving both normal and disordered functions of the immune system. Some of the especially promising and potentially rewarding areas meriting attention and singled out for encouragement and development are summarized in succeeding sections.

Host defense and the body's state of immunity depend upon the structural functional integrity of a widespread network of independent but coordinated cellular units populating many organs and tissues and trafficking via blood, lymph and body fluids. Accordingly, coorinated function of the immune system in the maintenance of health requires regulatory controls to achieve appropriate and effective immune responsiveness to antigenic stimulation. By the same token, any deficiency of constituent cells or their products, or aberrant regulatory processes or influences can result in immunologically mediated diseases. In order to prevent, diagnose, control and treat immunologic and allergic diseases it therefore is important to continue to direct attention to studies concerned with the basic biology of the immune system and the pathophysiologic processes by which it is affected.

Studies of immune regulation are proceeding at three levels. At the level of the immunocyte we are concerned with studies of membrane receptors and the signals these cells receive when the receptors are affected by antigens. At the level of immunocyte subpopulations affected through coordinated functions known as "circuits" we are concerned with their identification through membrane markers, the specific means of intercellular communication by antibodies or secretory factors leading to the immune response. At the level of the total immune system we are concerned with the summation of action of immunocyte clones reacting to antigens either in a positive way generating immunity or in a negative fashion resulting in tolerance, the processes of clonal selection and clonal interaction constituting the physiology of the immune system. This concept of a large, self-stimulating and self-regulating unit established by a network of idiotypes provides the basis of pursuit of productive and rewarding research.

It becomes increasingly important to understand homeostatic mechanisms in immune regulation especially since amplification processes effected by selective cellular proliferation can lead to lymphomatous neoplasia and idiotype-anti-idiotype interactions with resultant autoimmunity and immune complex pathology. Any ability to modulate disordered immune function in disease may well depend upon further insights that can be gained into such regulatory mechanisms that may control levels of expression of different immunoglobulin subtypes, allotype regulation of the B lymphocyte antibody producing system, and T lymphocyte control and T-B cell interactions regulating the switch in class production of immunoglobulins.

Two of the most important technologic advances vastly expanding research opportunities and the clinical applications of immunologic approaches have been that of hybridomas and cell cloning. We are only beginning to appreciate the vast opportunities that can be pursued through techniques leading to the fusion of antibody producing spleen cells with immunoglobulin producing myeloma cell lines both in immunologic research per se and the application of immunologic assays to basic and clinical questions. By obtaining continuous cell lines capable of effecting immunologic functions and the production of effector molecules it is now possible to investigate many aspects of the biochemical and molecular basis of cellular interactions and immune responsiveness, to purify and characterize B and T lymphocyte products, and to generate large amounts of monoclonal homogenous antibodies against a wide variety of important antigens. Accordingly, there is justification for encouragement of efforts to effect continuous cultured cell lines capable of generating unlimited amounts of useful antibody reagents for indefinite periods of time. Hopefully, it will be possible soon to bypass dependence on animal immunization for immunocyte produced reagents and the difficulties inherent in attempting to work with only small amounts of heterogeneous molecular mixtures.

Our newly found ability to clone immunocompetent cells in culture and to recover and examine their products has enhanced basic approaches to the analysis of composition, function, and regulation of the immune system. This technique has emerged from experience gained in the culture and clonal analysis of neoplastic myelomas and more recently recombinant DNA technology. Through its application it may soon be possible to to surmount difficulties inherent in in vivo approaches to the study of immunocompetent cells which we can now appreciate to constitute hetergeneous populations, each highly specialized in the manner in which individual lymphocytes-monocytes express unique antigen-binding specifity and mount an immune response. Some of the direct gains in basic research have resulted in increased understanding of structure of the different classes of the immunoglobulin molecules, their synthesis, assembly and cellular secretions, and the setting up of probes to allow for analysis of immunoglobulin gene organization and expression. Additionally, we now can be provided with uniform populations of cells to study surface antigen markers, identification of different lymphocyte subpopulations, and the study of cell surface receptors and lymphokine secretions in immunoregulatory functions.

Some of the areas of investigation anticipated to benefit by analysis of cloning immunocompetent cells are concerned with both basic understanding of immune responsiveness and clinical application of these processes. These include development of culture systems to grow lymphocytes and immunoregulatory cells for focusing on phenomena of antigen processing, presentation and recognition, expression of antigen sensitive helper and suppressor lymphocyte subpopulation, T cell switching in immunoglobulin class production, and communication between immunocompetent cells and lymphokine activities at the clonal level. Once this basic understanding of the immune system becomes clarified, clinical applications can be foreseen. The ability to control enhancement, restriction or modulation of natural clonal expansion in response to antigenic stimulation will be of great practical benefit in the treatment of neoplasia, allergy, chronic infections, autoimmune disorders and the suppression of transplantation rejection processes.

It is both relevant and important that immunologic research continue to keep alert to advances in molecular biology. The application of this special area has expanded our knowledge of molecular mechanisms in such immunologically relevant areas as lymphocyte differentiation and cellular interactions that result in humoral and cellular immune responsiveness. We look for continuing insights to be derived from such studies as the genomic organization of immunoglobulin genes, mRNA biogenetic steps, and the molecular basis of cell differentiation, all based upon the investigative probes of molecular biology.

Since a multiplicity of functional events involving the network of cells and molecules that comprise the immune system are effected by interactions at the cell membrane, we recognize the need to focus research efforts on the study of membrane antigens. Investigative reports serve to emphasize that that consequences of cellular interactions mediated by membrane molecules concern almost every aspect of immune function and malfunction. Accordingly, the need to elucidate mechanisms of such membrane entities and events as molecular receptors, the nature of signal transmissions across the membrane and triggering intracellular events leading to immune responsiveness is recognized as a prerequisite to fully characterize immune disfunctions and to devise therapeutic measures to correct imbalances in these processes. We have been especially concerned with studies on structure and function of membrane antigens encoded by genes within the major histocompatibility complex (MHC) whose products are clearly involved in cellular cooperation and in the recognition of alloantigenic specificities by T cells. Important research, therefore, is being directed to the biochemistry of cell surface glycoproteins and their significance in recognition phenomena eventuating in the killing of virus infected cells by cytotoxic lymphocytes. Through basic research of this character, potential clinical applications of basic information in this area can be clearly foreseen.

The chemical identification of lymphocyte products, i.e. lymphokines, has launched this field of investigation into a more rigorous and scientific phase. Notable has been the purification of interferon and colony stimulating factor. Development of methodology for large scale production, quantatative assays, and purification of lymphokines are being sought in order to pursue requisite studies to delineate the roles of lymphocyte secretory products in host defense, tissue destruction and inflammatory and immunologically mediated pathogenetic processes.

Studies on interferons have proceeded far beyond the original concept of their protective effects in virus infection. We can now appreciate the regulatory effects of interferon on the immune system, on interferon related disturbances in diseases states and interferon regulation of natural killer (NK) lymphocyte cells involved in lysing virus infected and tumor target cells. Ongoing investigations suggest that there may well be a genetic basis for interferon and NK activity and for failure of either interferon production or inability of NK cells to respond to interferon regulation in turn predisposing to persistent virus infection, GvH reactions and dissemination of malignancies.

Studies on effector molecules and complement biology continue to be highly visable and productive. Insight into mechanisms of complement activation and the role of complement in pathologic and inflammatory processes has been enhanced by results of rigorous chemical and physical methods utilized to characterize the components of this system and its cascading effects. Demonstrable biologic activities of cleaved products of complement have been applicable to our understanding of inflammatory processes in immune mediated hypersensitivity and allergic diseases. Accordingly, focus should be placed upon investigative approaches to the design and production of anti-complementary agents which can affect precise steps in the complement cascade. The roles of components of the complement system as inflammatory effecting agents and their pharmacologic control can find useful application and relevance to endeavors in infectious, immunologic and hematologic disorders.

In the area of research involving mediators of allergic and inflammatory reactions, increasing information has been generated on the roles and biologic activities of histamine, eosinophil and neutrophil chemotactic factors, proteoglycans and number of enzymes acting upon Hageman factor pathways. The most recent reports have offered exciting leads on lipid mediators derived from the lipoxygenase cyclo-oxygenase pathways from arachidonic acid. This especially concerns the prostaglandins and SRS-A whose chemical structural has recently been defined. It is now evident that SRS-A is actually comprised of a heterogeneity of biological molecules. Arachadonic acid of phosophlipid origin derived through the action of phosolipase becomes further involved in one of two pathways: 1. chemotaxis and chemokinesis effected by prostaglandins, thromboxane and prostycline products, and 2. the leukotrienes (SRS-A) now appreciated to result in bronchoconstriction and increased permeability of the microvasculature. As a result of these definitions we now understand at what point the antiinflammatory actions of corticosteroids, aspirin and indomethacin occur. Anaphylotoxins (C3a,C4a,C5a) are now understood to have genetic relationships, have both shared and distinct biologic actions, and exert nonhistamine mediated effects in lung tissue. Platelet activating factor, another lipid mediator, also has been characterized and synthesized and in experimental models in lung tissue. Platelet activating factor, another lipid mediator, has also been characterized and synthesized and in experimental models can mimic all the effects of an IgE mediated allergic reaction. Correspondently important research areas will involve identification of metabolic pathways and products in order to provide requisite information for the synthesis of analogs and development of functional antagonists in the treatment of allergic and inflammatory disorders.

While the proteins of the coagulation-kinin system have been highly purified and their molecular mechanisms of activation eluciated, definition of Hageman factor dependent pathways and their roles in the pathogenesis of allergic and inflammatory processes is still not clear. Since a role for these proteins is seen in the pathogenesis of such immunologic diseases as rheumatoid arthritis, glomerulonephritis and inflammatory skin diseases, potentially important and rewarding areas for clinical investigation present in this area.

Much information has also been elucidated concerning effector molecules involved in cellular inflammation, i.e., chemokinetic and chemotactic factors. The chemical definition of two, C5a and the N-formylated peptides provide prototypic approaches for study. The receptors for these two defined chemotoxins allowing for quantification on human neutrophils and evidence of functional abnormalities derived from receptor aberrations are recognized. Abnormalities in these proteins and humoral and cell factors which alter the ability of inflammatory cells to respond to chemotactic stimuli have been demonstrated in association with disease processes. Extended studies involving further chemical characterization of chemotactic factors and evaluation of their exact pathogenetic roles are providing investigative approaches for study of modulation of cellular inflammation by the design of appropriate pharmacologic agonists especially in the use of the arachidonic acid metabolites.

In the field of allergic disease research considerable progress has been made on understanding the nature of the IgE receptor with evidence suggesting that potential antiallergic intervention is possible at the level of peptide receptors blockage or on the function of the receptor unit. Additionally, it is shown that IgE synthesis may be governed by molecular interactions different from those that control other immunoglobulin classes. Selective helper and suppressor T cells and subsets offers productive leads in understanding and in turn possible management of asthma and atopic dermatitis.

Promising preliminary studies point to the feasability of modifying the chemical structure of causative allergens so that when utilized in immunotherapeutic procedures tolerance may be effected by suppressing ongoing specific IgE responses. Our best hopes for effective prevention, control and management of allergic patients lies in research directed to studies on the IgE receptor and receptor unit with the aim of intervention, the development of modified allergens to serve an tolerogens for IgE, and definition of suppressor and helper T cells and T cells factors that may offer leads whereby suppressor factors obtained from human lymphocytes might be employed to negatively modulate IgE production.

Another point of attack on allergic mechanisms is directed to elucidating biochemical release mechanisms of basophil and mast cell derived mediators. Since progress has been noted on the role of induced calcium influx, phospholid metabolism and changes in cyclic neucleotide levels, therapeutic approaches to control the secretory reaction may be possible. Accordingly, research is directed to the design of pharmacologic antagonists of specific pathways to selectively arrest secretory processes in mast cell and basophils based upon data derived from studies of the biochemical release mechanisms and the steps that ensue from receptor cross-linking. The relevance of these pathophysiologic processes to manifestations of asthma and urticaria gives reason to encourge such investigative approaches.

In the field of transplantation it is far from certain that matching at the A and B loci by tissue typing improves graft survival when donor and recipient of kidney are not living related donors. It now clear that matching at the D locus is particularly critical especially in bone marrow transplantation. While usable in renal allografting from a living donor, the time required in setting up MLC systems in D locus matching cannot be feasibly used in the case of cadaver donor kidney. Thus, studies on the role of the DR locus closely linked to the D locus in renal transplantation tissue typing show much promise. Additionally, except in the case of identical twins, there is increasing evidence that matching at the MHC locus alone does not guarantee graft survival. To be taken into consideration are other loci and various chromosomes which code for cell-surface antigens which could conceivably function as determinants of histocompatibility. Accordingly, alternative maneuvers to improve graft survival are under study including pretreatment of cadaveric kidney with corticosteroids and cyclosporin to reduce immunogenicity. Preculture of thymic cells and pancreatic islet cells has been found to sufficiently enhance survival after implantation. Whether prior blood transfusions have measurably beneficial effects and/or lead to sensitization of the recipient to a variety of donors is still not clear. One of the more exciting breakthroughs in transplantation biology has been the crossing of the species barrier in the implantation of the insulin producing islets in an experimental animal model of diabetes. The ultimate research goal will be to discover if similar islet incubation preculture at low temperature combined with a single dosage of the immunosuppressing agent will eventually permit successful transplants into human diabetic patients.

In view of the virtually near impossibility of effecting true histocompatibility, research efforts are directed to manipulation of the immune system of the recipient. Immunosuppressive drugs, cytotoxic agents and adrenal corticosteroids remain the mainstays of such immunosuppressive therapy. An alternative strategy under investigation involves massive irradiation of recipient lymphoid tissue followed by bone marrow transfusion. The goal of tolerance is being sought in post-irradiated states in which lymphoid organs are subsequently reseeded with lymphoid cell precursors. Additionally, specific immunosuppressants targeted at lymphocyte subpopulations, e.g. by the cyclosporin family of drugs, remains under potentially productive study. It is hoped that in the near future a careful tailoring of immunosuppressant treatment of the transplant recipient should become possible. Together with careful selection to avoid transmission of potentially lethal viral infections, e.g. cytomegalovirus, with the transplanted organ and pretreatment of the recipient the status of solid organ transplantation may be expected to improve.

Asthma continues to be an incompletely understood multifactorial disease necessitating investigative attention directed from several different approaches. Important factors for study are the physicochemical character and control of mucous secretion, protective role and possible alterations in bronchial mucosal barriers and bronchial epithelial permeability, structure and function studies involving mast cell distribution and smooth muscle of bronchi, role of neurogenic influences in the the control of bronchial smooth muscle tone effected through the autonomic nervous system and cholinergic activity, nonadrenergic inhibitory nervous influences in the tracheobronchial tree, and specific afferent receptors involved in the physiology of reflex mechanisms. Improved methods for the treatment of asthma are being designed on the basis of study of the pathophysiology of airway obstruction, neurogenic control of bronchial muscle and relevant pharmocodynamics. Promising studies underway include induced tolerance to aspirin in the intrinsic asthma syndrome, elucidation of pathogenic factors of bronchial hyperreactivity, food and environmental restrictions in infancy, natural history of asthma, role of low molecular weight industrial chemicals and air pollutants, and the effects of immunotherapy on established asthmatic patterns.

Tests of predictive value are required for a complete understanding of causes and management of drug-sensitivity reactions. In addition to identification of actual haptenic chemical determinants, in vitro immunologic detection systems should be sought. There is some preliminary suggestion that identification of HLA and other genetic markers may be associated with allergic reactivity based on altered drug metabolism, thereby offering hope for preventive approaches. Also under study are models to induce tolerance and the design of tolerogenic analogues of therapeutic agents in order to bypass administration of those with allergic potential.

Additional evidence has been offered to suggest that atopic dermititis once believed to be solely based on immediate hypersensitivity may actually involve complex etiologic mechanisms including T cell abnormalities and immunodeficiency factors. On this multifactorial basis continuing investigation is being directed to promising areas concerned with genetic predisposition and cAMP cell biologic functions interacting with IgE mechanisms. The importance of immune mechanisms continues to be demonstrated in a variety of connective tissue, cutaneous, renal, gastrointestinal, endocrine, hematologic and neoplastic conditions. Accordingly, encouragement is given to immunopathologic experimental approaches to the study of rheumatoid arthritis, inflammatory bowel disease, intestinal malabsorption syndromes, neurologic disorders such as myasthenia gravis and multiple sclerosis, glomerulonephritis and interstitial nephritis, systemic lupus erythematosus, juvenile diabetes, and lymphomas, Significant advances in basic immunology are reflected reflected both in increased understanding and in specifically designed investigative approaches involving this broad spectrum of immunologically related diseases. Improved technology allows for specification of etiologically important antigens and a definition of disordered immune pathways, e.g. immunodeficiency, immune complex deposition and autoimmunity. In earlier sections, mention was made of newer developing research areas relating to the identification and functional delineation of subpopulations of lymphoid cells, cell surface receptors, membrane antigens, immune regulation and gene coding for the MHC and its products. It becomes increasingly important that research not only be encouraged but that we also remain alert to research breakthroughs and promising leads that can be applied to solving clinical problems. We, therefore, are committed to directing our programmatic concerns to basic research in immunobiology, immunochemistry and immunogenetics technicologic advances offered by hybridoma and cell cloning procedures along with the tools of molecular biology, and properly designed and controlled clinical and investigative studies in order to develop potentially rewarding strategies in our attack on immunologic and allergic diseases.

Allergy and Clinical Immunology Branch

A. Scope

This Branch is concerned with the etiology, pathogenesis, diagnosis, prevention and treatment of allergic and immunologic diseases.

Studies of allergic diseases supported by the Branch include: (1) factors which contribute to asthma, such as extrinsic allergens, infections, abnormalitites of the sympathetic nervous system, chemical mediators of inflammation, and pharmacologic agents; (2) immediate type hypersensitivity and its disorders (allergic rhinitis, atopic dermatitis, urticaria and angioedema); (3) allergic phenomena affecting respiratory, gastrointestinal and cutaneous tissues; (4) allergic disorders caused by insect bites and stings, foods, airborne allergens, and infectious agents; (5) humoral antibody, particularly IgE, and the chemical mediators released by the interaction of antigen and antibody on target cells; (6) therapy and prevention of allergic disorders and hypersensitivity reactions; (7) manifestations of delayed hypersensitivity and contact dermatitis; (8) mechanisms of drug reactions and chemical sensitization; (9) isolation and chemical characterization of the active fractions of known allergenic agents; and (10) epidemiologic and environmental studies designed to ascertain those agents or substances which may be of clinical relevance to allergic individuals.

In the area of immunologic diseases, the Branch activities are focused on studies of the underlying mechanisms of disease and the application of basic knowledge to the etiology, prevention and management of immunologic disorders. Studies in clinical immunology are directed toward acquired and inherited diseases associated with dysfunctions of the immune system. Immunopathology studies include genetics, cytology, biochemistry, physiology and pharmacology of the immune system. Areas supported by the Branch include studies of (1) immune deficiency diseases arising from primary defects in development or maturation of the immune response or secondarily resulting from disorders affecting immune responses, (2) clinical manifestations mediated by products of lymphocytes, (3) diseases associated with immune complexes and autoimmune phenomena, (4) immunodermatology, i.e., immune disorders involving the cutaneous system, and (5) immunotherapy of disease processes.

The ultimate goal of the Allergy and Clinical Immunology Program is to promote the acquisition, translation, and application of research findings to the diagnosis, prevention, and treatment of allergic and immunologic diseases. To achieve this goal, the Branch is responsible for and manages Programs of Institute Emphasis (PIE) in Asthma and Allergic Diseases, Immunologic Diseases, and Immunology Centers and Program Projects. Collaborative clinical as well as basic research studies are supported among Centers and Program Projects in an effort to take advantage of the nation wide character of these programs and to facilitate achieving our goals. To support these efforts, the Branch funds targeted contract efforts and also obtains and distributes selected research reagents and materials to investigative allergists and clinical immunologists.

B. Awards and Support Levels

Allergy and Clinical Immunology

FY '80 Awards

Award Mechanisms	Number	Amount*
Research Grants	158	$17,358,592
Career Awards and Career Development Awards	15	580,747
Subtotal	173	$17,939,339
Fellowship Awards	12	164,766
Training Awards	22	1,662,077
Subtotal	34	$ 1,826,843
Contracts	9	0
Total	216	$19,766,182

*Total Costs, indirect costs estimated

The distribution of these awards by area of study is approximately 30% for asthma and allergic diseases and 70% for immunologic diseases. Approximately 34% of these awards were for competing new or renewal applications; the remainder represents commitments to support awards made in prior years.

Support for the PIE activities is included in the research grant category. During FY '80, the Branch supported these activities with awards for 17 Asthma and Allergic Diseases Centers at a total cost of $2,484,491, 6 Clinical Immunology and Immunopathology Program Projects at a total cost of $3,450,514, and 4 Centers for Interdisciplinary Research in Immunologic Diseases at a total cost of $1,261,556.

C. Program Areas and Highlights

1. Asthma and Allergic Disease Centers Program (AADC)

A network of 17 Asthma and Allergic Disease Centers, located throughout the United States, is actively engaged in both fundamental and applied research. Through this multi-faceted approach, methods have already been developed to improve the diagnosis and treatment of asthma and other allergic diseases. Although the emphasis of the Centers Program is directed toward a better understanding of the basic mechanisms involved in the various allergic disorders, they also have provided the field of allergy with unique academic resources in the discipline of allergy. By serving as referral centers for patients in their areas, by engaging in collaborative clinical trials to assess diagnostic and therapeutic discoveries, and finally by acting as an important resource for training academic allergists for the future, they have had a profound impact on the delivery of health care to allergic individuals. Separately each of these Centers have also been successful in competing for additional support through either the regular grant program, training grants, or research career awards.

During the past year, in an effort to additionally promote and support research in immunodermatology, two new centers whose predominant forms of investigation will be in dermatologic disorders were just funded. That brings to five the number of AADC Centers focusing on allergic dermatologic problems.

The 9th Annual AADC Workshop in Bethesda on November 29-30, 1979 was expanded to include not only AADC representative but also those from our Centers for Interdisciplinary Research in Immunologic Diseases (CIRIDs) [vide INFRA] and Program Projects in Mechanisms of Immunologic Diseases [vide INFRA]. About 125 participants met at NIH not only to review research progress by grantees but also to explore further potential areas of collaboration that might lead to more rapid translation of research findings to the bedside. As a consequence of this successful meeting, an Investigator Exchange Program was initiated for this year that will enable key members of these centers and programs to visit each other in a further attempt to promote meaningful collaborative exchange. A further highlight of this meeting was the exchange of views on promoting community activities relevant to persons suffering from allergic and immunologic disorders.

Brief highlights of some of the activities conducted by our Asthma & Allergic Disease Centers, not covered in last year's report follows:

K. Frank Austen, AI 10356, Harvard University, and his colleagues purified human C3 nephritic factor (C3 Nef) showing that it represents an autoantibody directed to C3b. Bb which functions to stabilize this amplification C3 convertase. They have improved the management of patients with hereditary angiodema due to C1 esterase inhibitor deficiency by short term use of antifibrinolytic agents (Tranexamic acid) in relation to oropharingeal surgery and especially by introduction of every other day therapy with attenuated androgens (oxymethalone) at a dose which both prevents attacks and minimizes undesirable side effects.

They experimentally elicited cholinergic urticaria in seven individuals by running on a treadmill in a plastic occlusive suit and showed not only the production of pruritic tiny skin wheals with macular erythema, but also changes in lung function, proving for the first time that this condition is not restricted to the skin. Finally, they have been extending knowledge of pulmonary changes by studies which use dispersed and concentrated human pulmonary mast cells, examining ultrastructure and characterizing granule components. For example, SRS-A generation was found not to parallel histamine release in such preparations. Of further interest is that rat SRS-A acts preferentially on peripheral, as opposed to central guinea pig airways *in vivo* and *in vitro*.

Alan P. Kaplan, AI 16337, SUNY at Stony Brook, NY, and his colleagues studying the pathogenesis of urticaria, angioedema and vasculitis, have identified patients with a new and previously undescribed form of physically induced urticaria which they have called cold-induced cholinergic urticaria (to be distinguished from cold urticaria because of their negative ice cube response).

Peter Kohler, AI 12028, University of Colorado, and colleagues studing allergic vasculitis, have made the provocative observation of an association between hyposensitization treatment (immunotherapy) with the development of systemic arteritis in 6 of 21 patients with polyarteritis nodosa. Five of the 6 also had circulating immune complexes. If confirmed by others, these studies would have important implications not only for the potential adverse consequence of immunotherapy but also the role of immunotherapy in causing vasculitis.

Elliott Middleton, Jr., AI 14198, Children's Hospital of Buffalo, and colleagues have shown that troleandomycin treatment of patients with severe asthma may work by inhibiting corticosteroid elimination. They have also made the provocative demonstration of an autoantibody directed against beta-adrenergic receptors in 3 normals, 4 asthmatic, and one patient with cystic fibrosis. Whether or not this is the cause of adrenergic hyperesponsiveness remains to be elucidated.

John H. Vaughn, AI 10386, Scripps Clinic and Research Foundation, and colleagues studying Aspirin Sensitive Asthmatics, have developed a safe way to challenge such individuals and have preliminary evidence to show that daily aspirin ingestion may actually improve symptoms in some of these patients. Further studies of the efficacy of an antihistamine/prednisone pretreatment regimen in persons allergic to radio contrast media, who needed repeat studies, showed that 238 procedures were carried out among 201 high risk patients with minimal and non-life threatening reactions (not requiring treatment) occuring in only 17 instances. Finally, they have extended previous observations concerning exacerbations of urticaria induced by oral administration of potassium iodide (KI) showing that dermal vasculitis and hypocomplementemia are clinical markers for possible KI sensitivity. These workers hypothesize, that the FDA currently under-estimates the number of individuals potentially at risk for iodine reactions; information of relevance if this drug is to be massively used in relation to nuclear plant accidents.

Robert G. Townley, AI 12029, Creighton University, and colleagues in their continued studies of the natural history of asthma were able to show that among families in which more that one member had asthma, positive methacholine responses pre-dated the onset of asthma, thus demonstriating for the first time the association of increased mecholy sensitivity with asthma. If confirmed among random populations, then mecholyl bronchoprovocation testing could be utilized to prospectively identify persons who are at risk to develop asthma. In other stuides they have shown for the first time that steriods produced an increase in beta-adrenergic receptors in rat lung membranes. This finding led them to postulate that steroid treatment in asthma may restore sensitivity to catecholamine, by increasing the density of beta-adrenergic receptors.

2. Centers for Interdisciplinary Research in Immunologic Diseases (CIRID)

In September 1978, 4 Centers for Interdisciplinary Research in Immunologic Diseases were funded by NIAID. This program is designed to foster integration and coordination of research projects in clinical immunology being pursued in other clinical specialties (of dermatology, pulmonary medicine, hematology, nephrology, rheumatology, infectious diseases and otorhinolaryngology) with those in basic research (e.g., immunochemistry, microbiology, virology genetics, biochemistry, pharmacology, general physiology, and pathology). Furthermore, study of one or more allergic diseases is a necessary component. An important additional component of these Centers is the funding of specific projects in a variety of community activities which are designed essentially to impact on health care in a tangible, and immediate way. It is hoped that these efforts along with the traditional basic research components, may provide guidance and future direction in how to best accomplish this transfer of technology.

During the past two years, these centers have matured substantially. At UCLA, John L. Fahey, AI 15332, and his colleagues showed that under physiologic conditions degensitization of immediate hypersensitivity could be achieved *in vitro* by IgE cross linking. It was also shown that during chronic oral therapy with long-acting beta-adrenergic agonists, normal subjects (but not asthmatics) exhibited a mild reduction in bronchodilator responsiveness and that a subset of asthmatic subjects developed increased sensitivity to the induced bronchospasm - a potentially clinically deleterious effect - a state from which recovery was hastened by administration of cortisosteroids. In other studies of patients with common variable hypogammaglobulinemia following tetanus immunization, their B cells failed to show spontaneous antibody production. Studies of the immune response to opportunistic infections have shown that 90% of patients with advanced cystic fibrosis had elevated levels of circulating immune complexes (CIC) and studies are underway to characterize the nature of these CIC. Interesting studies of aspergillus antibodies by an ELISA technique offers some promise in identifying patients with invasive disease, thereby avoiding hazardous surgical diagnostic techniques.

Continued evaluation of a supplementals program of non-medical intervention on the course of childhood asthma has shown patient acceptance and in preliminary results statistically siqificant improvement in the "treatment" groups was seen. Efforts are underway to collaborate and expand these studies with other investigative groups. A series of CIRID Innovative Efforts Awards were initiated to provide "seed" money and as well stimulate interest in relevant immunology investigations - Three were awarded including studies on Myasthenia gravis and thymic myeloid cells; and cellular immune response to auto-antigens in herpes simplex keratitis. This effort shows how other important disciplines can be encouraged to interact with immunology within an institution.

John P. Leddy, AI 15372, University of Rochester, and his colleagues have been following children prospectively after respiratory syncytial viral infections of the lower respiratory tract and interestingly have shown that 60% of 28 children have had episodes of wheezing subsequent to this infection. This may be an important factor in subsequent development of asthma in children. Taking advantage of their resource capability, the Rochester group has been performing, the immunological assays among a large group of individuals experiencing Kawasaki disease, a potentially fatal febrile illness of young children.

Charles W. Parker, AI 15322, Washington University, and colleagues have continued studies demonstrating the efficacy of oral desensitization of penicillin sensitive individuals; 30 such patients were able to successfully receive penicillin therapy. Continued studies indicated that arachidonic acid (AA) is a metabolic precursor of slow reacting substance of anaphylaxis (SRS-A) in guinea pig lung, that a lipoxygenase acting selectively at the S position of AA is probably involved and that SRS formed by immunologic and non immunologic means was chemically similar. With the recent elucidation of the struitme of SRS, studies such as these which may tell how to regulate its, production etc, and will be important in allergic individuals.

Joseph A. Bellanti, AI 15321, Georgetown University, and colleagues have continued efforts to develop safe effective live vaccines for bacterial pathogens by using temperature sensitive strains of H. influenzae as a prototype. Other studies have investigated the immunology of Langerhans cells in cutaneous and other disorders as well as the effects of steroids on alveolar macrophage function.

All 4 of the CIRIDs have continued their community activity programs.

3. Program Projects in Mechanisms of Immunologic Diseases

Program Projects were established to encourage the development of collaborative basic science and clinical investigation in immunologic diseases. As such this program is designed to further investigate underlying mechanisms of disease and to enhance basic knowledge relevant to eiology, prevention and management of immunologic disorders. Clinical immunology studies are directed toward acquired and inherited diseases associated with dysfunctions of the immune system. Immunopathology studies include specific areas of genetics, cytology, biochemistry, physiology, and pharmacology of the immune system and its disorders.

Highlights from those programs not referred to last year follows:

Hugh O. McDevitt, AI 11313, Stanford University, and colleagues have expanded their studies on the use of total lymphoid irradiation (TLI) in treating patients with severe rheumatoid arthritis unresponsive to other forms of treatment. Additionally this modality has found some potential role as an adjunct to treatment regimens in persons receiving transplants not only of Kidneys but also bone marrow. It is expected that this group will be collaborating with others during the forthcoming year to elucidate the potential role of TLI in clinical medicine.

In other studies they have shown that Shigella flexneri 2a and flexneri 1 cause Reiter's disease whereas Shigella sonnei does not. Analysis of these organisms are underway in an effort to develop specific diagnostic tests as well as learn how blood cells of patients with and without arthritis react to different components of these organisms.

Harvey R. Colten, AI 15033, Boston Children's Hospital, and associates have continued their studies of human serum complement genetics confirming that the liver was the major site of snythesis *in vivo* of C8, factun B, transferrin and other plasma proteins but not IgG. They have identified DW2 homozyogus specificities in families with C2 deficiency. Finally, they showed that C5 deficiency among 5 mouse strains was due to failure of secretion of C5 protein and not failure on biosynthesis of pro C5.

4. Mechanisms of Allergic Diseases

Studies within this program have been concerned with investigations of the etiologic factors, pathogenetic mechanisms, diagnostic measures, and therapeutic approaches related to the effective management of allergic disorders.

The release of inflammatory molecules from tissue mast cells is a necessary step in the evolution of allergic reaction of the immediate type. It is interesting to note that such reactions may also be relevant in other immune responses, such as those implicated in graft rejection, poison ivy and immune kidney diseases.

For example, Timothy J. Sullivan, AI 13880, University of Texas at Dallas, an allergic Diseases Academic Awardee has continued studies attempting to elucidate how mast cells release their chemical mediators found that diacylglycerol lipase, a heretofore unknown enzyme, resulted in release of fatty acids from mast cell membranes. The compound ETYA acts in part by inhibiting the action of this enzyme. It is hoped that such studies will lead to development of pharmacologic agents to act against allergic reactions.

Kimishige Ishizaka, AI 11202, AI 10060, Johns Hopkins University, found that bridging of IgE receptors caused methylation of phospholipids which resulted in uptake of calcium ion and subsequent histamine release from mast cells.

Marshall Plaut, AI 12810, Johns Hopkins has continued studies assessing the effects of histamine on lymphocytefunction by studying its effects on cytotoxic T lymphocytes in the mouse showing they lose their capacity to be inhibited when exposed to histamine. This establishes that regulation of inflammatory and immune response can occur both by the amount of each substances available and by altered responsiveness of the cells to these agents.

J. Andrew Grant, AI 12621, *University of Texas, Galveston*, during the course of continued investigations of basic function of basophils and mast cells reported a patient with hemophilia who experienced an IgE meidated allergic reaction to factor VIII infusions. They also studied a form of urticaria that was found to be mast cell rather than complement mediated.

The important role of IgE, or reaginic antibody, is well established in allergic responses. The means to regulate this response has been under intensive study by several groups. Kimishige Ishizaka, AI 14784, *Johns Hopkins University*, has been studying those mechanisms of regulation selective for IgE, rather than common to regulation of all immunoglobulin synthesis. Using an *in vitro* model of rat lymphocytes from lymph nodes injected with *Nippostrongylus brasiliensis*, he found an IgE potentiating factor derived from FC receptor bearing T cells. Studies are under way to learn whether this may be similar to the enhancing factor described by David Katz, AI 13874, *Scripps Clinic*. Dr. Katz own studies have continued and he has recently shown that *in vitro* synthesis of IgE in human lymphocytes closely follows observations made in animal systems. For example, using this sensitive assay system his group has shown that human IgE response can be specifically inhibited by tolerogenic compounds such as DNP-D-GL.

Alec Sehon, AI 14526, *University of Manitoba*, has continued an interesting series of observations attempting to induce tolerance, thus he was able to show suppression of IgE antibody response in mice to benzyl penicilloyl group (BPO) by adminstering BPO along with synthetic polymer (PVA). These conjugates ablated only the specific IgE response to BPO and none other. By expanding these studies to include allergens (ragweed) to conjugates of polyethylene glycol (PEG) a non immunogen in man, it is plarned to test this concept by using Ragweed-PEG conjugates in man.

Ross Rocklin, AI 15912, *Tufts University*, in a prospective study of patients with allergic rhinitis undergoing immunotherapy showed that antigen-specific suppressor mononuclear cells, likely bearing histamine receptors were generated during desensitization to allergy. This study represent an important step forward in investigating immune mechanisms that explain therapeutic efficacy of immunotherapy.

5. Asthma and the Other Allergic Diseases

Studies within this program have been concerned with investigations of the etiologic factors, pathogenetic mechanisms, diagnostic measures, and therapeutic approaches to the effective management of various allergic disorders. Among some of the diseases covered are asthma, allergic rhinitis, hay fever, hymenoptera sensitivity, drug allergy, as well as basic studies in allergic dermatitis, urticaria and other cutaneous disorders.

Approximately 9 million Americans have asthma, which in essence, constitutes a disorder of reversible airways obstruction. This obstruction has three major elements: 1) contraction of bronchial smooth muscles, 2) increased or retained secretions of mucus, and 3) inflammation of the respiratory tract mucosa. In individual asthmatics attacks can be provoked by exposure to specific allergens, and some of these individuals may have other allergies such as allergic rhinitis. On the other hand, many asthmatics do not have a demonstrable allergy, and in these individuals a variety of infectious, nervous (adrevergic and cholinergic), genetic or inflammatory factors may play a primary predisposing or contributory role. The program on asthma is structured to investigate all of the major parameters contributing to describing the mechanisms of causation in this disease. Areas under investigation include: the natural history, allergens, broncho-provocation challenge, animal models, pulmonary function testing, chemical meditors, cell mediators, agernergic agonists and antagonists, parasympathomemedic agents, drug allergy, diagnostic methodology, and therapeutic agents.

William W. Busse, AI 00343, University of Wisconsin (an Allergic Diseases Academic Awardee), has continued investigation of the mechanism by which viruses relate to the development and precipitation of asthma. They have shown that Rhino virus 16, a freguent viral isolate during respiratory illness provoked asthma, when incubated *in vitro* with human granulocytes, reduced responses to: isoproterenol, histamine, prostaglandin E. were demonstrated. Studies to determine how viral infections may alter beta adrenergic receptor function in asthmatics continue also in other labs (supra vide, section 1).

Studies continue in an effort to clarify the meaning of methacholine airways responses to indthacholine as well an allergen challenge in asthmatics. At Johns Hopkins, (AI 10304), James E. Fish examined the acute and delayed effects of inhaled ragweed antigen on airways tone and reactivity in hay fever and asthmatic individuals - finding that ragweed extract produced equally severe bronchospasm in both groups. They concluded that seasonal ragweed asthma is not explained by antigen induced changes in airway reactivity to non specific stimuli. Additionally they showed that in most asthmatics I.V. bolus of aninophyllin did little to prevent and did not immediately reverse antigen induced bronchospasm.

Roy Patterson, AI 11403, Northwester University, and colleagues showed that ragweed sensitive subjects had greater bronchial sensitivity to ragweed pollen after the season than before, so called "priming effect".

Finally, if the studies of Townley and colleagues (AI 12029) (supra vide section 1) Creighton University, are confirmed in the general population, then bronchial chellenge with mecholyl can become a method to detect the pre-asthmatic person.

Ragweed allergy is a disorder affecting approximately 15 million Americans. For most individuals it is a mild seasonal disorder commonly called hay fever, which is characterized by eye irritation and nasal discharge; symptoms are usually relieved by antihistamines; however, in some individuals is a source of considerable morbidity. In others ragweed has been indicated as being causal in producing an asthmatic-like syndrome. Most of the advances in understanding allergic rhinitis have occurred in this model, particularly in the areas of etiology, diagnosis, pathogenesis, treatment and prevention. Further progress can be expected to lead to greater understanding of factors involved in all allergic diseases.

Pertinent in this regard is the difficulty in obtaining purified allergen preparations. Despite the fact that there are at least 100 different proteins in ragweed, only five have been demonstrated to produce allergy in man. Several investigations have continued efforts at further puritication of and definition of the chemical structure of ragweed (J. Donald Capra, AI 12796, University of Texas at Dallas; T.P. King, AI 14422, Rockefeller University, and David G. Klapper, AI 14908, University of North Carolina, Chapel Hill). With further elucidation of structure, more intersting studies of how to alter allergens in such a way as to increase their efficacy and reduce side effects become possible. Thus T.P. King has prepared antigen E polymer conjugates which have been used by the Johns Hopkins group in treatment regimens. Roy Patterson, AI 11403, has shown efficacy of glutaraldehyde polymerized whole ragweed extract. Philip S. Norman, AI 10304, and his workers have prepared allergoid from common hay fever grasses treated with formaldehyde which are undergoing clinical trials.

Finally Arthur Malley, AI 13875, Medical Research Foundation of Oregon, has prepared antigen fragment protein conjugates of timothy grass to accomplish protection while minimizing side effects of immunotherapy.

It is obvious that clarification of the structure of individual allergens is important not only to improve chances of developing safer and more efficacious immunotherapy for allergic individualy but also in helping to perform these studies aimed at elucidating mechanisms of action.

Commercial suppliers of pollen for use in human desensitization work may vary in their source, so if an individual is receiving treatments in various parts of the country, there may be increasing or decreasing response to treatment. In addition and more importantly, if an allergist should change the supplier of his pollen extracts or if the supplier should obtain pollen from a different region of the country, the potency of the extract might be markedly different. Efforts are underway in this program to provide standardized antigen materials when possible, so that studies can be conducted among different groups.

Important with regards the latter point was the recent collaborative study guided by Tom van Metre at Johns Hopkins (AI 10304) which compared different methods and doses of administration of ragweed pollen extract. Showing that standard dosage schedule were superior to both Placebo and the Rinkel method of immunotherapy which delivers much less total dose of allergen, in relieving symptom of ragweed, hay fever.

Exciting recent advances have been further carried forward in the field of the sting of insects of the bee family called Hymenoptera, which induce an overwhelming and sometimes fatal reaction in sensitive individuals. Until recently the only materials available for testing and treating patients allergic to insect stings were so-called whole body extracts which were prepared by grinding up insects. Through the efforts of Lawrence M. Lichtenstein, AI 02870, Philip Norman, AI 10304, Martin Valentine, AI 15443, Johns Hopkins University, it was established that whole body extracts, available to practicing allergists contain little if any active vemon protein, and that desentization therapy with these extracts have virtually no utility in diagnosis or prevention of insect sting allergy. Subsequently, these investigators obtained venom itself, when administered in a series of immunotherapy injections to sensitive individuals, protection against serious reactions ensued in well over 95 percent of individuals. Other investigators have confirmed the efficacy of these materials, and in the spring of 1979, owing largely to their work, venom for desensitization was in fact made commercially available. Since that time they have determined that the clinical efficacy of venom immunotherapy is dose dependent being 80% at 50mg maintenance levels and 98% at 100mg. Even this regimen is imperfect and Gerald J. Gleich, AI 11483, Mayo Foundation have developed a program of passive immunotherapy for the 5-10% of patients in their study who failed to gain profective immunity in the usual way. These patients receive a single infusion of hyperimmune plasma from commercial beekeepers, and then receive deliberate honeybee stings to further stimulate their own active immunity.

Honeybee bites are not the only clinical problem and several groups, (Lawrence M. Lichtenstein, AI 08270, Johns Hopkins, and J. Andrew Grant, AI 12621, Unviersity of Texas at

Galveston) are attempting similar studies using Polistes Venom (WASP) to expedite progress in this area. Roger A. Morse, AI 16011, Cornell University, has been collecting a variety of vespid species venom.

Because of this institute's interest in immunodermatology several of our grantees have concertrated efforts on a variety of allergic dermatologic conditions. William L. Weston, AI 16637, University of Colorado, has examined 16 patients with erythema multiforme noting that in 13 instances the E.M. was preceded by skin lesions compatible with herpes simplex viral (HSV) infection. This had led to focus on HSV - associated E.M. to learn whether there is a causal association.

Robert E. Jordon, AI 14550, Medical College of Wiconsin, and colleagues have been systematically studying persons with chronic vesiculobullous skin disease. Among persons with pemphigus, the identification of auto antibodies to proteins located between individual epidermal cells in host skin has led to improved diagnostic tests. In bullous pemphigoid, they postulate that interaction of auto antibodies with antigen in skin tests to activate complement and cause blister formation.

Jon M. Hanifin, AI 15557, University of Oregon Health Sciences Center, studying receptor pharmacology in Atopic Dermatitis found that in vivo desensitization by inflammatory mediators (e.g. histamine) could be the cause of beta blockade seen in atopic dermatitis.

Henry C. Maguire, AI 13337, Hahnemann Medical College and Hospital, has shown that in patients who have a subsiding allergic contact dermatitis, when reexposed to the same allergen (patch testing) often reactivate sites of prior resolving contact dermatitis, (so-called flare reaction) is due to T cells in the sites (not antibody mediated).

Vera S. Byers, AI 12947, U.C. at San Francisco, extended her studies of poison oak immunity, which have shown that urushiol, the oil of the plant, poison oak/ivy, contains a very small chemical which is unusually fat soluble, explaining its easy entry into skin and body. By developing analogues which may be carried to the site of entry by for example, low density lipoproterns, she hopes to develop blocking compounds which could be used therapeutically.

N. Franklin Adkinson, Jr., Johns Hopkins University, has continued a series of basic and clinical studies of penicillin allergy (AI 15525, AI 11936). He has found that patients with a history of prior penicillin allergic reactions who have currently negative skin tests to minor determinant mixture have a risk of developing an acute reaction if retreated (1-2%).

Furthermore refined RAST testing has identified more than 85% of penicillin allergic patients. One finding of some interest was that 14.9% of patients who had positive history of penicillin allergy but negative skin tests actually experienced reactions on receiving penicillin therapy, fortunately mild. Finally that the problem remains serious is illustrated by one patient who had an anaphylactic reaction to the skin test only (there was a failure to use prior scratch test).

6. Immunodeficiency

The program on immunodeficiency is concerned with the etiology, ontogeny, prevention, and treatment of structural and functional deficiencies of the immune system. Both naturally occurring and acquired disease states are included. Ongoing studies have focused on the congenital absence, failure of development, or other disorders affecting thymus or bone marrow cellular elements; abnormalities in production, inhibition or catabolism of immunoglobulin; deficiencies of specific complement components; and defective host defenses due to abnormalities of leukocytes. These naturally occurring or acquired defects of immunity provide a unique opportunity to expand the understanding of normal immune function. Only by its absence do we see a role for its presence in the sequence of steps which make up the normal immune response. Although these diseases are rare, the information gained is relevant to the general field of immunology and to many health problems including infectious dieases, allergy and arthritis.

Pertinent to immunodeficiency studies are elucidation of normal acquisition of immunity. E. Richard Stiehm, AI 07008, UCLA, and colleagues are evaluating the feasibility of using gramma globulin in oral feedings of small newborns as a substitute for antibody rich breast milk, other studies have shown that newborn lymphocyte may produce a soluble suppressor factor when exposed in vitro to certain plant lectins, and could explain the reduced ability of the newborn to fight off infection.

Donald G. Hanson, AI 13474, National Asthma Center, have shown that if normal newborn mice are fed a potential protein allergen prior to subseguent exposure parenterally by injection, they develop tolerance and no allergy to this material. Elucidation and recognition of ways to take advantage of this observation may lead to ways to reduce childhood allergies. Richard M. Rothberg, AI 07854, Unviersity of Chicago, and colleagues are also studying immune responses of newborn to ingested antigens.

Ralph J. Wedgwood, AI 07073, University of Washington, in continued studies of common variable immunodeficiency have shown that excess suppressor cell function was important in few patients. Efforts to alter immune function in such individuals include studies by Sheldon Horowitz, AI 15723, University of Wisconsin, who is examining the potential therapeutic role of thymosin on T cell function. In conjuction with Richard Hong, AI 14354, University of Wisconsin, one child treated with cultured thymic epithelial transplant was shown to develop suppressor T cell function *in vivo* which ablated a comob's positive hemolytic anemia. An interesting series of observations have been made by Robert A. Good, AI 11843, Sloan-Kettering, and colleagues who showed that T cell abnormalitites in individuals with common variable immunodeficiency improved after treatment with dietary zinc supplements. They have also studied the secondary immunodeficiency in cancer patients caused by zinc deficiency. Details from animal studies to clarify these findings included 1) zinc deficiency in animals led to decrease thymic hormone level and deficiency of natural killer activity against leukemia cell lines; malfunctions which were corrected by giving zinc. 2) studies showing that dietary restriction prevents breast cancer in mice. Because marrow transplantation and other efforts at immune egulation and reconstitution are imperfect in their effects, every possible avenue of investigation need be explored.

7. Immune Complex Disease

Immune complexes result from the binding of antigens by antibodies. Subsequently, components of the complement system also become involved. Such complexes, therefore, arise during the course of the development of the normal immune response. Under some circumstances, however, these entities appear within lesions and indeed are necessary for the development. The pathogenetic mechanisms by which immune complexes cause tissue damage, particularly in the auto-immune diseases with which they are increasingly associated are still poorly understood. The lack of sensitive, quantitative assays for all types of immune complexes has impeded work in this area to some extent.

William P. Arend, AI 10975, University of Washington, has made an effort ot determine what it is that renders an immune complex damaging. He reports that in rheumatoid arthritis, anti-IgG antibodies (rheumatoid factor) specific for the FC portion reacted with IgG to produce innocuous complexes, whereas anti-IgG antibodies specific for the FAB portion produced injurious complexes.

Systemic Lupus Erythematosus is an auto-immune disease in which anti-DNA antibodies play a permanent pathogenetic role. The cause for the appearance of the auto-antibodies is being studied in a genetically defined mouse strain which develops an

SLE-like syndrome spontaneously. Defective suppression of the immune response in these animals can break the self-tolerance usually seen (M. Eric Gershwin, AI 00193, University of California, Davis). Many people are studying the strain and have shown that the induction of tolerance to DNA can be accomplished by treatment with appropriately constructed antigen. Bernard Stollar, AI 14534, Tufts University, has undertaken studies to define the chemical requirements for developing reagents that can specifically prevent the formation of antibodies to nucleic acid. In detailed studies of the specificity of tolerance, in studies done in collaboration with Yves Borel, AI 13867, Boston Children's Hospital Medical Center, he has shown that tolerance to one nucleoside does not prevent the formation of antibodies to other nucleosides of DNA. This finding provides an approach to the study of the regulation of responsiveness and specific non-responsiveness at the cellular level. The obvious hope in this regard is to develop information sufficient to permit appropriate manipulation of the immune response in order to effect therapeutic benefits. Thus, the development of specific immune tolerance would be a significant improvement over the use of non-specific immunosuppressive or anti-inflammatory therapy in the treatment of auto-immune disorders.

8. Inflammation

The process of inflammation is mediated by cells and by humoral factors. This process is essential for host defense against foreign substancs. However, in certain disease states, inflammation plays a major role in tissue damage. An understanding of what initiates and what modulates inflammation is, therefore, crucial to explaining and controlling these disease states. The noncellular mediators of inflammation can be divided into two general categories, those of small molecular weight and those of high molecular weight. The group of serum proteins derived from the complement system and the associated enzyme systems of coagulation, fibrinolysis, and kinin fall into the latter category.

The initiation of the complement cascade by either the classic or alternate pathway makes a major contribution to the development of the inflammatory response. Once activated, complement components have functions including opsonization, immune adherence, chemotaxis, cell lysis, and anaphylatoxin generation. Derangements of complement have been implicated in causing or contributing to a wide range of disorders including cancer, kidney diseases, collagen vasular diseases, infectious diseases, and inherited diseases.

Understanding of the complement system depends on isolation and identification of its components. It is known that there are approximately 20 complement components. Paul A. Liberti

(AI 12365, Thomas Jefferson Unviersity) has devised an isolation procedure for C12 which results in stable prepartions retaining all native biological activities. Irma Gigli (AI 13809, New York University) has shown that Cm serves an important function in the initial steips of complement activation and that C4bp functions in the control of the formation of C42 convertase, confirming the presence of a control mechanisms in serum which regulates the classical pathway of convertase.

Particular interest has been placed on studies of the properdin or alternate complement pathway. The alternate pathway was, until recently, thought to have specific recognition proteins which bound to and thereby identified foreign particles for destruction. Hans Muller-Eberhard (AI 07007, Scripps Clinic and Research Foundation) reports that the complement protein, C3, has recognition function in the so-called alternate pathway. Antibody is not required fo rhtis reaction. The deposited C3 interacts with surface structures of activators such that a regulatory protein which ordinarily initiates inactivation of deposited C3, cannot reach it. As a consequence, the orderly assembly of complement enzymes procedes without interference by regulators and the cell is killed by the membrance attack complex. It is of interest that this could have relevance in cancer study, and in fact, Alfred Esser, AI 14099, Scripps Clinic and Research Foundation, has shown that certian RNA tumor viruses can be lysed by human complement, supporting the proposition that complement can provide a major defense against infection with reto viruses (oncornaviruses, C-type viruses, mouse leukemia viruses which may be associated with the production of tumors in animals).

These basic studies have been very rewarding because they are useful in explaining mechanisms for several clinical problems. K. Frank Austen (AI 07722, Robert B. Brigham Hospital) reports C3 nephritic factor, the abnormal protein present in patients with membranoproliferative glomerulonephritis, causes the complement system to be fully activated in the serum of patients with various types of nephritis. This protein is a host antibody directed against protein of the alternate complement pathway and serves to stablize the interaction of the complement protein thereby circumventing all host regulatory mechanisms. This leads to virtually complete depletion of the complement system with resultant inflammatory reaction. He is working on the role of the alternate complement pathway in bullous pemphigoid, a disease in which the mast cell effector system also appears to be abnormal.

Dr. Gigli states that recent studies have shown that the complement system may play an important role in the localization of myocardial infarction. Depletion of one of the components, C3, is accompanied by better outcome of animals in which experimental infarcts have been induced. Kenneth Mathews, AI 14950, University of Michigan, Ann Arbor, has found that deficiency of serum carboxy-peptidase B, which might predispose to radiographic contrast media reactions or chronic idiopathic urticaria or angiodema (worked on in collaboration with Tony Hugli, AI 15782, Scripps Clinic). The potential significance of this observation is that there may be a subpopulation of patients who not only react adversely to radio contrast dyes, but who also may have the syndrome of chronic urticaria or angiodema, where serum carboxypeptidase B deficiency may contribute to their clinical difficulties.

Other mediators of inflammation include slow reacting substance of anaphylaxis (SRSA) which was recently identified as a leukotriene and whose availability in pure form will now permit a series of studies isolating its importance in asthma where it is thought to play a major role in bronchospasm.

A discussion of basophils (or mast cells) must accompany any discussion of these chemicals, because these are the cells which release them. The sequence of events leading to mediator secretion is as follows; sensitization takes place; IgE attaches to the surface of mast cells and basophils; on re-exposure, the cell-bound antibodies are bridged by the allergen signaling release of the mediators from the cells.

Research in this area has been facilitated by several new advances: Herve Bazin (AI 12830, Catholic University of Louvain) through the development of a colony of rats with IgE secreting plasmacytomas, has made this immunoglobulin readily available; and both Dr. Parker and Dr. Ishizaka have established rat basophilic leukemia cells in culture.

Both of the latter investigators are also studying basophil receptors. Dr. Ishizaka as been able to demonstrate histamine release from these cells via divalent anti-receptor antibody without participation of IgE. Also studying these receptors in Floyd J. Malveaux (AI 05383, Johns Hopkins University) who has noted the relationship between serum IgE and the number of basophil IgE receptors.

While Dr. Ishizaka has shown that mast cells can discharge allergic mediators without involvement of IgE, Lawrence M. Lichtenstein (AI 11334, Johns Hopkins University) has demonstrated release of mediators from other than mast cells. Namely polymorphonuclear (PMN) leukocytes contain and release slow reacting substance of anaphylaxis (SRS-A) and eosinophilic chemotactic factor (ECF).

Philip S. Askenase (AI 12211, Yale University) is continuing study so-called cutaneous basophil hypersensitivity (CBH).

Traditional views of delayed hypersensitivity and immediate hypersensitivity as being separate patterns of immune reactivity may no longer hold. For example, otherwise typical delayed reactions contain significant numbers of basophils. CBH can be transferred in animal models by either immune cells or serum. Similarly, the late IgE mediated reactions in man may be analogous to serum transfer of CBH.

Finally, Eosinophils are known to be involved in inflammatory processes, particularly those allergic in nature. For this reason, some mention should be made of research in this area. Gerald Gleich (AI 09728, Mayo Clinic) has localized the basic protein of the eosinophil to the core of the granule, called major basic protein (MBP). He has demonstrated that MBP is toxic to schistosomules of Schistosoma nansoni and newborn larvae of Trihinella spiralis. Furthermore he has developed a radio immuno assay for human MBP finding it in serum and correlated with blood eosinophilis. Finally it has been shown increased in sputum of asthmatics which could be an important source of injury to bronchial epithelium in such individuals. Elucidation and extension other observations are experted to clarify questions in in both allergic and parasiti disorders.

Genetics and Transplantation Biology Program

A. Scope

This Branch supports and manages research on the immunologic factors that determine the acceptance or rejection of grafts and on the genetic relationships that underlie them.

The increasing prevalence of degenerative disease and of trauma that may impair the function of particular organs sufficiently to be incapacitating or lifethreatening identifies the perfection of a successful transplantation methodology as one of the objectives of highest priority in current medical science. The technical surgical aspects have proved quite manageable but immunological rejection of the graft is the obstacle that has yet to be surmounted.

Research efforts supported by this Branch include:

1. Studies of strategies designed to minimize the likelihood of rejection by assuring the best possible antigenic match between donor and recipient.

2. Explorations of immunosuppressive regimens whose objective is to reduce the capacity of the host to reject the graft.

3. Studies of treatments of the graft that will reduce its ability to provoke a response.

Studies in animal models are supported that explore the genetic controls of the immune response, and, on a more practical level, test methodologies whose ultimate application is to man.

Genetically conditioned associations between antigenic characteristics and susceptibility to various diseases have recently been brought to light and also fall within the purview of this Branch.

The Serum Bank is an important distinct component of the Branch. It is a international resource, providing sera and homozygous typing cells for research on human immunogenetics and for histocompatibility testing performed in conjunction with organ transplantation. The continuing evolution of the Bank is currently taking it into information gathering and analysis in the field of population immunogenetics.

B. Awards and Support Levels

The Genetics and Transplantation Biology Branch supports research activities through individual grants, research career development awards, fellowships, training grants, and very importantly, in connection with its organ transplant program and serum banks, through contracts.

The following shows the distribution of support for the activities of the Branch during FY 1980.

Genetics and Transplantation Biology Branch

FY '80 Awards

Award Mechanism		Number	Amount*
Research Grants		58	$ 6,467,622
Career and Career Development Awards		9	337,024
	Subtotal	67	$ 6,804,646
Fellowship Awards		10	127,387
Training Awards		1	22,703
	Subtotal	11	$ 150,090
Contracts		24	1,929,217
	Total	102	$ 8,883,953

* Total Costs, indirect costs estimated

The majority of these awards, approximately 80%, are concerned directly with transplantation biology and the remainer support research on the genetics of the immune system and of disease associations. Approximately 30% of these awards were for competing new or renewal applications; the remainder represents commitments to support awards made in prior years.

C. Program Highlights

1. Transplantation

(a) The Kidney Transplant Histocompatibility Study

Data on all phases of renal transplantation, collected in the course of a multicenter observational study conducted in 1974-1978, were subjected to intensive analysis during the past year by the Branch with the collaboration of the Naval Medical Research Institute (NMRI)(YO1 AI 50001). This resulted in a publication comprehensive in scope describing the donors and recipients, the practice of transplantation and the results achieved at the participating hospital centers. This publication is obtainable from the Government Printing Office.

A total of 2,418 kidney transplants were studied. In 885 of the cases the donor was living and related to the recipient and in 1,533 the donor was unrelated and had died suddenly, permitting the removal of a viable organ. The initial objective of the study had been to determine what effect the matching of the antigens coded by the A and B loci of the donors and recipients has on the probability of long-term survival of the graft. However, to permit adjustment for the effects of complicating factors (confounding variables), information was also gathered on virtually all aspects of the "natural history" of the transplants. Such a large, comprehensive data base permits the effective application of multivariate analysis and an excellent statistical model was developed for this purpose by NMRI. In addition, the subjects, procedures and outcomes were characterized by means of frequency tables, and correlations among the various characteristics and between subject and procedure characteristics and outcomes were explored in actuarial life tables.

Many of the results of the analyses are highly interesting and instructive, although at this stage they should be viewed with caution because sufficiently extensive dissections of the major predictors of outcome have not yet been carried out.

The known better survival of grafts taken from living, related donors is well-documented: 49% of kidneys from cadaveric donors remain functioning one year after engraftment while 73% of the organs given by relatives of the recipients remain so. Equally impressive is the fact that death of the recipient occurs in 19% of the former and only 10% of the latter during the first year. Thus, the living related donor transplant is far more successful than the cadaveric donor transplant. This is also reflected, but much less dramatically, in the level of rehabilitation attained at one year in patients who have retained a functioning graft. Overall, 71% are fully and 85% substantially returned to normal lives. Among recipients of cadaveric grafts, 67%, and among recipients of grafts from living donors 77% are capable of normal, full-time activity. The significance of these statistics lies in the support that they give to the utility of transplantation as a treatment of end-stage renal disease.

In contrast to the utility of tissue typing in living donor transplants, its efficacy in cadaveric transplants is highly dubious, especialy if allowance is made for differences in the races and in the number of pretransplant transfusions given recipients matched to different extents at the A and B loci.

The great preponderance of donors was white (88%), with blacks being the other well-represented group. Of recipients of cadaveric kidneys, however, 20% were black and 79% were white. The race of the recipient was very significantly predictive of outcome: 52% of white recipients of cadaveric grafts retained them at 1 year, vs 38% of the black recipients. The basis for this dramatic difference is not yet clear. That is, it has not yet been determined whether this difference is the result of the differences in causes of renal failure (there is a higher incidence of hypertension in blacks, of diabetic glomerulosclerosis in whites), of selection and treatment procedures, or of some other factors. This matter is now under investigation. What is known is that (a) the higher incidence of graft failure in blacks does not result from a higher mortality rate, and (b) it is associated with a lower rate of decay of the slightly higher excess initial risk attendant on transplantation in blacks. This last is revealed through the application of the statistical model which divides the risk of graft loss into an excess initial risk and a long-term risk, and characterizes the duration during which the former has a bearing by means of a parameter defining its rate of decay.

Among the manipulations that affect the survival of the graft two stand out as very clearly beneficial. The first is blood transfusion which can increase 1-year graft survival by nearly 20% (37% in the absence of transfusions prior to the transplant, to 56% if the recipient was given 6 or more transfusions). This observation must, however, be approached with some caution because transfusions, while substantially reducing the excess initial risk, do elevate the long term risk. This late burden appears to be associated principally with large numbers of pretransplant transfusions. Indeed, the range 6-10 seems to be optimal.

The powerful beneficial effect can also mask the contributions of other factors with which it tends to be associated. Thus, for example, the poor performance of subsequent transplants, after the first had failed, is not evident unless allowance is made for the fact that previously transplanted persons are more likely to have been transfused.

A very nearly equally dramatic improvement in cadaveric-donor graft survival is effected by pre-transplant splenectomy. This is the result of a profound reduction of an albeit somewhat more persistent excess initial risk, and also of the long term risk. The impact of splenectomy is robust and survives adjustment for the effects of the recipient's age (graft survival decreases with increasing age, but only in the case of cadaver, not living, donor transplants) race, transfusion history, diseases causing renal failure, and pretransplant nephrectomy, all of the recipient. Bilateral pretransplant nephrectomy appears at first sight to be as effective as splenectomy, but its statistical significance is lost when allowance is made for its association with splenectomy and with specific diseases causing renal failure. Thus, splenectomy, but not nephrectomy, appears to have an intrinsic beneficial effect.

Among other predictive factors usually considered significant are the diseases leading to renal failure. Thus, diabetic and hypertensive nephrosclerosis are ominous. The former is prominent in whites, the latter in blacks. Indeed, the statistical significance of the effect of diabetic glomerusclerosis is sharpened when allowance is made for this difference in distribution among the races. Also noteworthy is the concentration of this effect on the long-term risk of graft failure.

Retention of the graft is critically dependent on pharmacologic immunosuppression of the recipient. The standard agents used are the corticosteroids and azathioprine. Anti-lymphocyte/thymocyte serum/globulin was used in various centers also. A clear case for its efficacy in cadaveric graft transplants can only be made if it is used in the immediate perioperative period. Interpretation of this observation is complicated by the great variability in the preparations of this agent and in the manner it was used by the various hospital centers. Similarly, the dramatic beneficial effect of the use of heparin at the time of transplantation is completely confounded by the fact that it was used essentially only at one of the major centers and there it was administered to nearly all transplant recipients.

The "center effect" was deliberately avoided during the first phase of KTHS data analysis. It is currently being addressed in the second phase through a contract with the Peter Bent Brigham Hospital and the Harvard School of Public Health (NO1 AI 96235). One very interesting result obtained to date is that the dramatically better results obtained at one major transplant center could be accounted for in terms of objective predictors of graft survival identified in a multivariate analysis based on a statistical model distinct from the one developed by NMRI. On the other hand, treatment of all the major centers in this way revealed that although many of the disparities in performance could be predicted from the practices identified in this way, residual differences remain. In other words, the "center effect" has not yet been adequately explained. These investigations are to be published soon in Transplantation Proceedings (Analysis of NIAID Kidney Transplant Histocompatibility Study (KTHS); Factors Associated with Transplant Outcomes, I., by B. A. Barnes and D. Olivier).

The culture of epidermal cells, renal epithelial and mesangial cells, and vascular endothelial cells in pure form has been made practical. Not only are culture techniques of high reliability available, but procedures have been developed to unambiguously identify the proliferating cells and to establish their similarity to, or differences from, the tissues that served as their sources.

The newly available monoclonal antibodies with exquisite and selectable specificities also promise to substantially facilitate research on organ-specific antigens.

Given our knowledge of evolution and molecular biology, any protein synthesized by a cell must be considered potentially polymorphic and therefore to be capable of functioning, if it is expressed on the cell surface, as a transplantation antigen. This includes the differentiation antigens transiently expressed on the cell as it matures. Indeed, the absence of polymorphism must be imposed by severe evolutionary and adaptive constraints that require conservation of structure.

One task facing the field is to explore the extent of this polymorphism, that is the number and frequency of the alleles, the distribution of the antigens on the various types of a given tissue such as vascular endothelium, and, more generally, the pattern of their expression during differentiation and aging. It is in the performance of this task that the combination of cell culture methods capable of yielding abundant quantities of antigens, starting with a well-defined "ancestor", and the technology for the production of monoclonal antibodies specific for these antigens and capable of locating and identifying them in situ in the tissue should prove singularly useful.

A second task is the broader characterization of the role of organ-specific alloantigens in precipitating allograft rejection. Here again, the availability of antigens and target cells obtained by culturing, against which to test the sera and cells of graft recipients should be helpful. But here also the classical methodologies retain their utility. Among the specific questions that require answers are: (1) Is there potentiation of rejection when disparities in the MHC and in other alloantigens coexist? (2) In what cases is recognition of organ-specific alloantigens MHC-restricted? (3) Is rejection of non-MHC "minor" or of organ-specific antigens more susceptible to suppression with pharmacologic agents? (4) Is there transient expression of differentiation antigens in the engrafted tissue and can it precipitate rejection or even autoimmune processes?

A Request for (Grant) Applications was issued to coincide with the above conference and six applications were submitted in response. They are now under review. It is evident that the problem of minor and tissue-specific antigens is becoming tractable as more effective technologies are coming into general use. If attention continues to be drawn to this field, substantial progress may be expected.

(c) Immunosuppression

Acceptance of allografts, i.e. graft tissues of donors on recipients who are not identical twins, is critically dependent on the modification of the response of the host's immune system. The ideal form of unresponsiveness is antigen-specific, leaving the host's immune system capable of coping with infectious agents and altered endogenous antigens such as those on tumor cells. Research whose objective this is is underway, focusing on utilization of antiidiotype reactivity (Gordon, Colorado, AI 16825) and on pharmacologic agents capable of suppressing or destroying antigen-stimulated lymphocytes (cyclosporin A may act this way). However, as such approaches are not yet fully clinically practical, the mainstays of routine immunosuppression are the well-established drugs, the adrenal corticosteroids and the cytotoxic agents, which unfortunately also have broad effects on various organ systems. Although the need to tailor the schedule and dosage of these agents is well-recognized and attempts to measure the depth of immunosuppression have been made (H. M. Lee, Medical College of Virginia, AI 12822, P.S. Russell, Massachusetts General Hospital, AI 07027), the analytic tools available to date have not been adequate to the purpose.

The recent advent of lymphocyte subset-specific monoclonal anitbodies capable of identifying not only the functional types of mononuclear cells (B lymphocytes, T helper and suppressor/cytotoxic cells, NK cells, monocytes, etc.) but also their state of activation and differentiation has made immunologic monitoring a practical undertaking.

The effectiveness of these reagents has been amply demonstrated in studies in the laboratory of S.F. Schlossman (Sidney Farber Cancer Institute, AI 12009) on the immunologic disturbances that may underlie various diseases. Thus acute graft-vs-host-disease (GVHD), an early and clinically homogeneous complication of bone marrow transplantation, is characterized by an absence of suppressor T cells in the circulation and the exclusive presence of activated helper T cells. The later-onset and more heterogeneous chronic graft-vs-host-disease displays a variety of disturbances in the balance of the T cell subsets. Similarly, in a case of multiple autoimmune disorders a cellular pattern comparable to that of acute GVHD was found, while in a case of acquired immunodeficiency, the defect could be localized on the predominance of activated suppressor T cells. In multiple sclerosis, an association was found between loss of suppressor T cells and the active stage of the disease. Very interestingly, the immunosuppression observed in viral disease, specifically infectious mononucleosis (EBV infection), was found to coincide with an increase in activated T suppressor cells, and recovery with return to a normal T cell subset balance.

Consequently, the Branch has initiated a study, under contract with Dr. S. F. Schlossman (NO 1 AI 02650) to investigate the balance of the lymphocyte subpopulations in patients subjected to pharmacological and physical immunosuppression to begin the analysis of the modes of action of these agents. The ones under study are the steroids, the cytotoxic agents, including azathoprine and cyclophosphamide, and lymphoid irradiation. Attention is focused on the use of these agents in renal and bone marrow renal recipients, although they are also being investigated in patients with systemic lupus erythematosus, with rheumatologic and atopic disorders, in some persons with melanoma with a small tumor load undergoing immunomanipulations.

It is expected that the procedures developed and the information obtained will permit the more precise adjustment of doses of conventional drugs used to condition the recipient and to control rejection postoperatively. It is also expected that the experience with the monoclonal antibodies and the understanding of the balance of the subsets needed to prevent rejection gained in the study will permit their being used to modulate the immune system.

2. Molecular Genetics

Knowledge of the molecular genetics of the immune system has expanded explosively in the past very few years and the way is open for major new advances. The technological underpinnings of this field are the techniques for sequencing and for cloning and expansion of segments of DNA, including genes, in bacteria, the ability to culture mammalian cell lines, some at various stages of differentiation, and the developing ability to genetically transform mammalian cells with or without the use of vectors.

The major advances made to date have been in the elucidation of the structure and function of the genes coding for immunoglobulins, for example in the laboratory of L. E. Hood (Stanford University, AI 10781), and have resulted principally from determinations of the sequences of the proteins and of the genes. The most salient observations are that the immunoglobulin genes are interrupted, that pieces coding the various regions of the proteins (exons) are dispersed on the chromosome and separated by untranslated sequences (introns), and, most important, that expression of particular genes is dependent upon rearrangements of the chromosome resulting in deletion of a number of intervening regions. Thus, in the case of the mouse of light chain gene, the leader (L) segment coding for the signal peptide is interrupted, resumes and there the sequence continues through most of the variable (V) region which is also interrupted and widely separated from its end, the J-segment. This segment is, in turn, separated from the region coding for the constant region. Differentiation from the embryonic configuration into that seen the mature, Ig producing plasma cell, results in the joining of a V region and a J region gene. Allelic exclusion, the failure of one of a pair Ig gene alleles to be expressed, appears to result from the failure to undergo such a rearrangement.

The organization and the rearrangements of the heavy-chain variables regions are still more complex. Again in the mouse, there appears to exist an additional region, the D segment, which must be joined to the rest. Moreover, there is a separate and distinct region for each of the domains of each class and subclass of the constant portion of the heavy chain, as well as a segment that codes for the polypeptide segment that is at the carboxy end of membrane-bound Ig.

The astounding diversity of Ig combining-region sequences, and consequently of specificity or antigen recognition, is beginning to be accounted for. Thus, there are several hundred copies of genes for the variable regions of light and heavy chains, and each may associate with several different J regions (and also D regions in the case of heavy chains). The joining process which occurs during differentiation can, itself, add to the diversity because the point of junction does not appear to be fixed, insertion or deletion of residues occurring. These events may be considered somatic mutations, but additional diversity may be introduced through more conventional somatic mutations independent of the joining process.

The joining of initially separated runs of nucleic acids also occurs during the maturation of RNA (Randolph Wall, AI 13410). This process excises untranslated intervening sequences coded by the introns and also play a role in differentiation. Thus, the secreted and membrane-bound versions of IgM are encoded by the same Cu gene. However, which of the forms is produced, and the relative amount produced, is dependent on the splicing that occurs, in one case leaving a segment coding for the 20 amino acids at the carboxy end of secreted IgM and in the other a membrane-binding 40 amino acids run. It is now evident that the splicing as well as the addition of the 5'-terminal cap and the 3'-terminal poly A are signaled by specific sequences on the primary transcript.

Important progress is also beginning to be made on other aspects of molecular immunogenetics. The tools and experience obtained in the course of studies of Ig genes, are being applied to investigations of the antigen receptor on T cells, presumably containing Ig heavy chain variable regions (L. E. Hood, AI 17565).

Work on the genes of the major histocompatibility complex (MHC) shows great promise. Sequencing of the proteins is progressing in a number of laboratories (S.G. Nathenson, Albert Einstein School of Medicine, AI 07289 , T. J. Kindt, NIAID, J. Strominger, Harvard University, AI 10736). In vitro translational systems utilizing MHC or RNA have been developed and etic isolation of the pure RNA is nearly completed (J. Strominger, see above). The importance of such an isolation results from the fact that with pure RNA, after reverse transcription and cloning, an exploration of the architecture of the MHC genes becomes possible.

Regulation of function of cells of the immune system can be approached in several ways. In one, cells may be transformed at various stages of differentiation into continuous cell lines to amplify the number of cells available for each stage. The genetic architecture of the stages then becomes accessible. In another approach, complementation of genes may be effected by fusion of cells with differing degrees of differentiation. Questions of dominance, of cis or trans effects, of structural vs. regulatory gene function can thus be answered.

A still more elegant procedure has now become available: cell-transformation using isolated DNA. The process is analogous to transformation of bacteria with DNA and results in the incorporation of the genes presented to the host cells as precipitated DNA and in their functional expression. Gene cloning technology permits the isolation and amplification of genes or DNA segments of interest and transformation permits their selective transfer. Transformation tends to occur at low frequency, necessitating the use of procedures capable of high selectivity. A general process is now available, co-transformation with a gene for drug resistance, because co-transformation occurs with a frequency of 80% of transformation itself.

With the two methods for effecting gene transfer established, cell hybridization and transformation with DNA, many questions pertaining to the control of gene expression and of cellular differentiation became accessible. It is clear, however, that to exploit these opportunities broadly, resources in the form of a broad range of established cell lines with defined function will be needed.

3. The Serum Bank

The mission of the Serum Bank has been to provide high quality sera and cells for tissue typing to support research in immunogenetics and transplantation immunology. The bank therefore acquired, through purchase and donations, sera recognizing antigens encoded by the A, B, C, and DR loci of the human major histocompatibility complex, and typing cells homozygous at the D locus, evaluated these reagents, and distributed them (the sera in bulk form and on the NIH typing tray) to basic and clinical investigators.

During the past year the Serum Bank underwent major administrative and conceptual restructuring. Much of the routine activity of monitoring the flow of reagents in and out of the Bank has been automated. Analysis of the evaluations of the tests performed by reference laboratories to determine the quality of the sera submitted has been recaptured. Firm policies regarding the distribution of sera, restricting the availability of the rare specifities and reemphasizing that the Bank exists to support research, were issued. A revised catalog supplement describing the sera in the Bank and specifying the restrictions on their distribution was published. As a consequence confidence in the Bank was restored, attested to by a great resurgence of offers of sera, including those specific for rare antigens. This permitted the addition of 42 liters of sera to the Bank's inventory, compared to an outflow of 13 liters, the average distribution in recent years, thus rebuilding the stocks and permitting the removal of several sera from the restricted category.

The redesign of the typing tray represents a major conceptual innovation. The standard tray had been replaced by a Basic and an Extended Tray system. The Basic Tray recognizes the commonly encountered antigens and the cross-reactive groups of the A and B loci. It should suffice in the great majority of typings for transplantation, especially those involving living related donor grafts, and will be available for research on an unrestricted basis. The Basic Tray was designed to achieve two purposes. The first, just stated, will result in the more economical use of rare sera and therefore will enable the Bank to make the trays more broadly available (heretofore they were distributed solely to support human organ transplantation). The second was to enable private organizations to produce it by including on it only the more readily available antigen specificities, and so to permit the Bank to, ultimately, cease producing it itself. This objective was announced to the tissue typing community and to organizations with a capability in this area. Several are prepared to accept responsibility for the Basic Tray, in toto, but further progress towards this end awaits delicensing of the sera and their regulation as "medical devices" by the Food and Drug Administration.

Recognizing, however, that on occasion, the Basic Tray will prove inadequate to ascertain an individual's transplantation antigens and that the rare sera needed to achieve this are not and will not be readily available, the Bank will continue to produce the companion Extended Tray. This tray carries sera for rare specificities and splits at the A and B loci and sera recognizing antigens coded by the C locus. This tray is produced in limited quantities and its distribution will be more closely controlled.

The Bank has also undertaken to produce trays to serve specialized needs. The first of these is the DR locus tray. A matter currently under intense study is the utility of matching at the DR locus in, especially, renal transplantation. The DR tray should provide a broadly available standard set of high-quality reagents to facilitate research on this question.

Additional trays are under consideration but their production hinges on the acquisition of appropriate sera. For example, it is generally accepted that blacks frequently cannot be tissue typed satisfactorily with the commonly available sera obtained, by and large, from non-black women (pregnancy serves to immunize). A workshop to define the magnitude of the problem and to indicate promising approaches is scheduled for September 1980. It is expected that it will be followed by a planned program for the acquisition and testing of sera recognizing black antigens and finally by a tray that will carry them and will be used to type blacks.

Reagents of another type are potentially of great use in human histocompatibility testing but sufficient experience with them is not yet available. These are the monoclonal antibodies produced by mouse hybridomas. To evaluate them the Branch has organized a workshop. The first step, to be taken in October 1980, will be to examine the various techniques that may be useful in their application as typing reagents and to select the most practical one. In the next stage, the 50 or so monoclonal antibodies recognizing human polymorphic cell surface antigens will be prescreened and then distributed to laboratories possessing well-studied families and broad population panels. In this phase the recognition patterns of these antibodies will be defined and compared to conventional sera.

The Serum Bank is also currently active in efforts to achieve greater standardization of current tissue typing techniques, in collaboration with the American Association for Clinical Histocompatibility Testing (AACHT), and in the development of a generally more reliable and less demanding technique (F.C. Grumet, Stanford University, NO1 AI 82558).

IMMUNOBIOLOGY AND IMMUNOCHEMISTRY BRANCH

A. Scope

This Branch is concerned with the biology and chemistry of the immune system and its products. The fundamental studies which it supports on the structure and function of the immune system are directed toward acquiring a complete understanding of immune response mechanisms at their basic cellular and molecular levels as they function in health and disease.

Activities in this broadly-based program area cross traditional disciplinary lines of biology and chemistry and encompass anatomic, physiologic, pharmacologic, and microbial biology and organic, physical, and biological chemistry. Its scope includes studies of the origin, maturation, localization, and function of immunologically active cell populations, and the mechanisms involved in the induction, modulation, regulation, and expression of immune reactivity, as well as physicochemical studies of antigens and their homologous antibodies and the mechanisms and kinetics of antigen-antibody reactions.

Research activites supported within this program area are grouped in the general programs for Immunobiology and Immunochemistry and in a Program of Institute Emphasis (PIE) for Lymphocyte Biology.

B. Awards and Support Levels

Relevant activities in immunobiology and immunochemistry are supported through various mechanisms including contracts, individual post-doctoral fellowships, institutional pre- and post-doctoral training grants, career and career development awards, as well as investigator-initiated research grants.

The following shows the distribution of support by award mechanism for the activities of the Branch during FY 1980.

Immunobiology and Immunochemistry Branch
FY 1980 Awards

Award Mechanism	Number	Amount[1]
Research Grants	230	$24,535,474
Career and Career Development Awards	23	840,195
Subtotal	253	$25,375,669
Fellowship Awards	30	330,120
Training Awards	9	668,531
Subtotal	39	$ 998,651
Contracts	4	30,009
Total	296	$26,404,329

[1] Total costs, indirect costs estimated

The distribution of these awards by discipline is approximately 3/4 for immunobiology and 1/4 for immunochemistry. Approximately 35% of these awards were for competing new or renewal applications; the remainder represents commitments to support awards made in prior years.

Support for the Lymphocyte Biology PIE is included in the research grant category. During FY 1980, five program project awards, at a total cost of $3,397,005, were made to support this activity.

C. Program Areas and Highlights

1. Program of Institute Emphasis for Lymphocyte Biology

This Program currently supports five program projects, each of which is a multidisciplinary research effort integrating the technical expertise of cell biologists, geneticists, cellular immunologists and immunochemists and is directed by an acknowledged leader in the field. Their combined studies are designed to expand knowledge of the morphologic and functional heterogeneity of lymphocyte populations and to develop the capability for identification and selection of lymphocyte subpopulations with specific immune reactivity or antigenic composition, for hybridization of such populations and for selective production of specific lymphocyte products. The objective of this Program is to acquire as complete a knowledge as possible of the life history of immunocompetent cells and of the physiologic and external factors that determine their fate and function in vivo and in vitro.

This Program was initiated approximately eight years ago with the award of four program project grants, each directed toward the goal of understanding the biology of the lymphocyte and having different but complementary approaches. Three of the original projects have successfully undergone competitive peer review and have received renewal of support for a five-year period. In addition, support for two new program projects has recently been provided, one each in FY 1978 and FY 1979.

The program project directed by Matthew D. Scharff (AI 10702, Albert Einstein College of Medicine) has focused its approaches on the molecular biology and somatic cell genetics of the immune system to define the genetic and molecular control mechanisms of immunoglobulin synthesis, the structure and synthesis of the antigens of the major histocompatability complex (MHC), and the functions and interactions of T and B lymphocyte populations and macrophages. In many of these studies, continuous cloned cell lines and variants derived from these cell lines are being used to study the role of the cells and their products in the immune response. For example, Dr. Scharff and his associates have devoted considerable time and effort to generate immunologically functional hybrids between mouse myeloma cells and spleen cells at a high frequency and some of the drug marked cells which are now widely used to prepare hybridomas originated in their laboratory. They have provided cell lines and assistance to well over a hundred investigators around the world to establish this technology in their own laboratories. Studies within the program project have been productive and important contributions have been made. Cultured mouse myeloma cell lines have been used to examine the genetic control of the expression of immunoglobulin genes.

Analysis of antigen-binding variants of these cell lines indicates that the variants arise spontaneously at a very high frequency and may reflect the normal process which regulates the generation of antibody diversity. Primary sequence analysis of these immunoglobulin variants has provided evidence that they have structural changes in the constant region of the heavy chain which cause major changes in the folding of the variable region and produce significant changes in antigen binding. Of interest is Dr. Scharff's recent observation that hybridomas producing monoclonal antibodies generate constant, and probably variable, region variants similar to those occuring in myeloma cells and with comparable frequency. Studies of the biochemical properties and primary structure of the H-2 MHC products, in an attempt to relate functional and genetic properties with molecular structure, have suggested that discrete amino acid changes in the H-2 glycoproteins are directly related to their immunologic specificity.

In order to pursue the mechanisms underlying the complex and coordinated functions of macrophages and to identify the molecular basis for modulation of these functions by products of activated lymphocytes, physiologic and functional studies of cloned macrophage-like cell lines have been initiated. An unusual macrophage-like cell line has been established which can migrate, phagocytize, and respond to the lymphokine, macrophage inhibitory factor (MIF), which affects the migration of normal macrophages. These, as well as other differentiated functions, have not been previously retained in other continuous macrophage cell lines. Because MIF is quantitated only by bioassay, this cell line should prove to be a valuable tool which will facilitate studies on the mechanism of action of MIF, as well as for studies of the functional role of macrophages in infection and immunity. Related studies of immunologic activation of macrophages and the products of activated macrophages have demonstrated that they play a key role in producing neurologic tissue damage as a consequence of inflammatory demyelination. Evidence has been obtained which indicates that the pathology of experimental allergic encephalomyelitis can be prevented or diminished by inhibition of neutral proteases secreted from activated macrophages or by interfering with the ability of macrophages to infiltrate the target tissue.

The functional status of immunoregulatory mechanisms in chronic infections also is being investigated. The anergy or immunologic unresponsiveness which is frequently observed in patients with lepromatous leprosy has been associated with the presence of suppressor T lymphocytes induced by antigens of the leprosy bacillus. Patients with tuberculoid leprosy exibit normal immune responses and do not possess such suppressor cells. The identification of the antigen-inducible suppressor cell as a T lymphocyte raises the possibility of effective treatment of lepromatous patients with specific immunoglobulins against this subset of T cells. Similarly, diminished antigen-induced suppressor cell activity was found in patients with multiple sclerosis and lupus erythematosus, both of which are suspected of being caused by persistent viruses. These patients also were found to be unable to produce leukocyte interferon and exhibited markedly reduced natural killer cell activity. Because addition of interferon to cell cultures was found to correct this defect, interferon may be useful in treatment of these disorders. Also, development of a laboratory test to screen such patient responses to interferon may provide a means to predict the results of interferon therapy in patients with these diseases.

The approach directed by Gerald M. Edelman (AI 11378, Rockefeller University) centers on the analysis of molecules and molecular assemblies involved in the control and recognition of antigenic stimuli at the surface and within the cells of the immune system. The results of his studies have provided several insights into the nature of recognition and control events in lymphocyte function. Of special interest is his evidence for the existence of a surface modulating assembly (SMA) which regulates cell suface receptors and mediates transmembrane signalling for functional cellular responses. Dr. Edelman and his associates picture the SMA as a three-component system consisting of cell surface receptors that penetrate the cell membrane, submembranous microfilaments which coordinate receptor movement, and microtubules which provide anchorage for the receptors and propagate signals to and from cell surface.

One long-term objective of this project is to reconstitute a functional SMA. For this effort, a method has been developed to prepare portions of surface membranes from lymphocytes. Using specially prepared beads and antibodies against surface molecules, lymphocytes can be bound to the beads and, after lysis of the cells, patches of membrane remain bound to the solid bead surface. Dr. Edelman and his associates have demonstrated that the inner surface of the membrane is exposed on these patches and that some cytoskeletal elements remain associated with them. Specific antibodies have been prepared against the transmembrane proteins so that their external and internal portions can be distinguished. With these antisera and the surface membrane fragments, it should now be possible to analyze the constitutents of the cell surface membrane complex and to define the mechanisms by which antigenic stimuli and other signals are transmitted from outside the cell through the membrane to sites of metabolic activity within the cell cytoplasm.

A viral gene protein has been established as a valid probe to dissect the stimulatory sequence leading from the cell membrane to the cytoplasm and the nucleus and to study the enzymes involved in cellular DNA replication. Using this approach, evidence has been obtained which indicates that the microtubules and microfilaments which form the cytoskeleton act as signal regulators in controlling cell growth and division. Related studies are focused on the enzymatic activities involved in initiation of chromosomal DNA replication and mitogenesis, as well as the factors which control them. An in vitro assay for DNA replication using a small, well-defined, DNA molecule as a template has been developed for this purpose. Preliminary fractionation of cell extracts has demonstrated that DNA replicative activity is contained in two fractions, each of which is required for full activity. Subfractionation efforts are now in progress to isolate, identify, and characterize the enzymatic proteins.

To further understand the nature of molecular interactions at the cell surface, physicochemical studies of cell surface molecules and of selected external stimuli also are being pursued. A major portion of this effort is concerned with the chemical structure and molecular interactions of the gene products of the murine MHC. Because these molecules are believed to serve as recognition units at the surface of immunocytes, attempts are being made to identify those portions which are involved in interactions with other molecules at or under the cell surface. The heavy chain of one H-2 molecule has been characterized in detail and compared with other murine and human cell surface receptors. Considerable homology between the murine molecules was noted, suggesting that they may be allelic products of the same gene locus. Evidence was also provided that the carbohydrate components of these molecules do not serve as alloantigenic determinants and apparently do not play a major role in the three-dimensional folding of the molecule. Of interest, however, is the observation that the heavy chain, as well as the beta-2 microglobulin light chain, contain residues which are phosphorylated. The role of the phosphorylation of these transmembrane molecules in their interaction with other surface, cytoskeletal, and cytoplasmic elements is now being examined to determine its involvement in signal transmission and response controls. To complement this effort and to gain an understanding of these molecular interactions, attempts are being made to define the three-dimensional structure of beta-2 microglobulin using high resolution X-ray crystallography. Although Dr. Edelman's research is centered on the role of the SMA in the immune response, the implication of his results are broad and suggest that the SMA has a key role in controlling cell movement, growth, and differentiation in general.

The program project directed by Jonathan W. Uhr (AI 11851, University of Texas Health Science Center at Dallas) is centered on the study of the biochemistry and biology of lymphocyte surface molecules. The structure of immunoglobulins on the membrane of B cells is being studied in order to clarify the molecular interactions that occur after surface immunoglobulins react with specific antigens. The broader purpose of such studies is to obtain an understanding of the molecular mechanisms which underlie signalling of B lymphocytes and control their differentiation. Studies of T cells emphasize the process of immune recognition; efforts are being made to biochemically characterize the T cell receptor and to define the role of Ia antigens in the interaction of antigen-pulsed macrophages with T cells. The major concern and principal contributions of Dr. Uhr's project have been in the area of the ontogeny, regulation, and function of B cell surface immunoglobulins. An immunoglobulin D (IgD)-like molecule was found on the surface of circulating murine lymphocytes; ontogenic studies showed that it appeared after immunoglobulin M (IgM) when mice were approximately one week of age. The results of additional studies have led to the acceptance of a model which assigns an important role for IgD in the development and function of B cells. In this model, immature B cells first develop surface IgM, then acquire surface IgD to become "double bearers"; some cells subsequently lose surface IgM to become cells that bear only surface IgD. Dr. Uhr and his associates have demonstrated that cells which bear only surface IgM become tolerized after encounter with antigens. They also have shown that surface IgD acts as a triggering receptor so that cells that bear both IgM and IgD give a primary IgM response after encounter with antigen; cells that bear only surface IgD give an IgG response after encounter with antigen.

In recent studies, he and his associates have employed two types of antigens which activate B cells to produce antibody by different mechanisms to further define the role of surface IgM and IgD. One type can stimulate B cells directly without requiring the help of T cells; the other type apparently depends on the active participation of T cells. From studies using antibodies against B cell surface immunoglobulins, they concluded that T independent antigens require only surface IgM to activate B cells while T dependent responses require both surface IgM and IgD. They have suggested that T independent antigens do not require the participation of surface IgD in B cell activation because they possess an appropriate mitogenic capability which could directly deliver a triggering signal to the cell.

In an effort to relate function to structure, comparative physicochemical studies of membrane and secreted forms of IgM have been continued. Recent data indicate that membrane IgM contains a hydrophobic peptide in the constant region of the carboxy-terminal end of the mu chain which is not present in the secreted form. Although the function of this peptide has not yet been precisely defined, its hydrophobic character suggests that it may have a role in anchoring the molecule in the cell membrane.

Studies of surface receptors on T cells have similarly led to important conceptual advances. Evidence has been obtained to indicate that specific binding of antigens coupled to autologous macrophages by T cells represents the initial event in immune stimulation. The macrophage plays a key role in this stage because T cell receptors do not specifically bind to native exogenous antigens. The results of recent studies suggest that fragments of exogenous antigen, presumably "processed" by the macrophage, and molecular products of the I region of the MHC are the principal molecules involved in lymphocyte recognition of the macrophage surface. They believe that the Ia molecule binds with, and determines the orientation of, antigenic fragments on the surface membrane and, in this manner, appropriately presents the immunodeterminant to the T lymphocyte. The results of additional studies indicate that the interaction between antigen-pulsed macrophages and T lymphocytes can be subdivided into a number of successive stages. Definition of physical parameters and chemical agents affecting these phases has led to the identification of molecular mechanisms underlying the specific binding of T lymphocytes to antigen-pulsed macrophages. Lymphocyte mobility has been demonstrated to be important in the early phases of such interactions, presumably by increasing the probability of effective contact between complementary sites on the surface of these cells. Treatment of cell cultures to reduce lymphocyte mobility, such as by reduction of incubation temperatures or with inhibitors of oxidative phosphorylation and glycoloysis, significantly reduces specific binding of T cells to macrophages. Once the cells have interacted, divalent cations are required to maintain stable binding, relying perhaps on the ionic forces generated by these cations at the membrane interface of the adherent cells. Stable interactions appear to be followed by transmission of stimulatory metabolic signals to the lymphocyte, leading to its activation, differentiation, and proliferation after dissociating from the macrophage. Lymphocyte activation also involves the participation of cations, particularly in early mitotic events. Of interest is Dr. Uhr's observation that lithium, an ion widely used pharmacologically in various psychiatric disorders and having immunologic side effects including lymphopenia, thymic involution, and the appearance of antinuclear antibodies, can modulate lymphocyte activation. Although inhibitory in some circumstances, lithium has been found to enchance lymphocyte activation when the cells are appropriately stimulated with mitogens.

The program project directed by Baruj Benacerraf (AI 14732, Harvard Medical School) is designed to gain understanding of the genetic controls of the immune system and of the regulation of functional expressions of immunocompetent cells. Component projects are concerned with the identification and function of immune response genes, the induction, regulation, and function of helper and suppressor T cells, the identification and function of surface receptors on T and B cells, and the definition of macrophage functions and interactions in the immune response. Using genetically-determined responder and nonresponder strains of mice, Dr. Benacerraf and his associates have found that specific suppression can be eliminated by administration of antisera specific for I-J gene-controlled determinants; immunopotentiation of nonresponder mice with this antiserum was demonstrated to reflect a reduction of specific suppressor responses.

The results of more recent studies have provided evidence that the antigen-specific factor derived from T cells of nonresponder mice may share idiotypic determinants, as well as anti-I-J determinants, within the same molecular complex. These results imply that there are shared variable regions on antibody heavy chains and on T cells which are crucial in the suppression pathway for the same antigen. Related studies on the mechanisms of nonresponsiveness have led to a clear distinction between T cell tolerance and T cell suppression. T cell tolerance exhibited a three-day induction period, had a high inducer cell dose requirement, and was not affected by pretreatment with cyclophosphamide. In contrast, T cell suppression required 7 days for induction, was induced by 1,000-fold fewer cells, and was inhibited by cyclophosphamide pretreatment.

In other studies, macrophages of responder mice were shown to be able to present antigen in an immunogenic form and to play a central role in regulating the balance of activated helper and suppressor T cells. These findings suggested that the predominant suppressor T cell responses in nonresponder mice may be associated with an Ir gene defect at the macrophage level. Of interest in this context is their demonstration that macrophages of neonatal mice do not present antigen efficiently to T cells and that only a small number of these macrophages bear Ia surface molecules. Thus, the ease of tolerance induction and the limited immune response capacity of newborn mice may reflect defective macrophage function but also may reflect a significant mechanism for control of self-reactivity during this developmental period. Hybridoma technology is being employed to gain further insight into macrophage surface components and their function. To date, three surface molecules have been identified by specific monoclonal antibodies. Mac-1 is a protein which can be detected on murine macrophages and granulocytes while Mac-2 and Mac-3 represent molecules which are distinct and present on macrophages but not on other leukocytes. Mac-1 also has been detected on human monocytes and granulocytes and on human and mouse natural killer cells. The availability of monoclonal antibodies against distinct macrophage surface components will permit more detailed studies of the role of these cells in infections and malignant diseases, as well as in the immune response.

Detailed analysis of the aggregation or capping of surface molecules on B cells during their activation is continuing, with particular emphasis on ionic changes. Recent data indicate that calcium ions are mobilized and released from the cell interior by a transmembrane process when mouse B cell surface immunoglobulins are bound by ligands. The calcium ions changes were not accompanied by a general ionic pertubation within the cell and did not depend on the extracellular ionic environment but was correlated with the cytoskeletal contractile activation which leads to capping of surface immunoglobulins. Mobilization and release of intracellular calcium, thus, may represent an early, physiological, ionic signal for capping. B cell capping also was found to be related to adenosine metabolism; treatment of murine or human B cells with combinations of coformycin, an inhibitor of adenosine deaminase, homocysteine, and adenosine impaired immunoglobulin capping. Inhibition of capping was also produced by 3-deazaadenosine, a specific inhibitor of adenosyl homocysteine hydrolase. The practical significane of these studies was established by demonstrating that patients with adenosine deaminase deficiency, a condition which is often associated with a generalized immunodeficiency, exhibited impaired capping of B cell surface immunoglobulins. Patients who had been successfully treated by bone marrow transplantation did not exhibit such an impairment.

The Program Project directed by Carl W. Pierce (AI 15353, Jewish Hospital of St. Louis) was initiated in FY 1979 and has the immunobiology of the MHC as its central theme. The combined efforts of Dr. Pierce and his associates provide a multidisciplinary approach to elucidate fundamental mechanisms of the immune response. Their studies are focused on the biochemistry, genetics, and functions of the MHC, the nature of receptors on immunocompetent cells, and in the diverse regulatory mechanisms of the immune system. Areas currently being examined include the nature of the interactions between histocompatibility gene products and conventional antigens in the generation of effector lymphocytes, the structure of genetically-restricted suppressor factors, the structure and genetic relationships of components of the complement system that are produced by genes of the MHC, the identification and structure of gene products on T cells and macrophages, and the identification and characterization of receptor molecules on the surface of T lymphocytes.

One approach to study the function of these gene products is to construct liposomes containing isolated H-2 antigens which would stimulate intact cells in various assay systems. At present, liposomes containing H-2 and Ia antigens have been constructed and tested; both types of antigens were demonstrated to be integrally inserted in the liposomal bilayer and to have retained antigenic reactivity in serologic and cytotoxic cell assays. In related studies, a human T cell proliferative assay system has been developed to study gene products of the HLA-D region, the analog of the murine I region. Using this system, preliminary results indicate that human responses are regulated and restricted genetically in much the same way as is the murine immune system.

Chemical differences between Ia molecules in murine macrophages and B cells also are being examined. Tryptic digests of Ia molecules from macrophages and B cells were examined by high pressure liquid chromatography. The beta chains of I-A molecules yielded identical peptide profiles but the alpha chain profiles showed clear differences which are now being studied to define their chemical nature and their possible functional significance. In an effort to obtain a sufficient supply of I region molecules for further study, attempts are being made to clonally propagate murine macrophages and to apply hybridoma technology. T suppressor cells have been successfully hybridized with a thymoma and large quantitites of antigen-specific, Ia bearing, suppressor factor are now being produced for biochemical and immunologic analyses. Preliminary results indicate that the hybridoma-produced suppressor factor has functional activity comparable to that produced by, or extracted from, suppressor T cells.

This Program of Institute Emphasis represents a major investment of the IAIDP and the NIAID in the concept that advances in the understanding of the behavior of lymphocytes can be effectively achieved through carefully organized efforts by large, highly interactive groups led by well-established and highly productive senior scientists. To date, there is every indication that this Program has been successful and productive and that it will continue so in the future. However, because the renewal cycle for these program projects will begin during FY 1982, preliminary plans for either continuation, modification, or an orderly termination of this Program will be developed during FY 1981 on the basis of Institute needs and resources.

2. Immunobiology

This program is concerned with the processes leading to immunocyte differentiation, proliferation, and production of biologically-active substances which mediate immune reactions.

Research supported within this program includes studies on the origin, maturation, and localization of immunologically active cell populations, the interactions of lymphocyte subpopulations and their interrelationships with macrophages and other leukocytes, the cellular phenomena of antigen processing, immunologic tolerance and enhancement, and the mechanisms involved in the induction, modulation, and regulation of immune responses. The cellular mechanisms involved in the induction, maintenance, expression and pathophysiology of delayed-type hypersensitive immune reactivity are also included in this category. Also relevant are studies of the lymphokines and other lymphocyte substances which activate macrophages and have pharmacologic effects on lymphocyte and other leukocytic cell functions such as chemotaxis, phagocytosis, blastogenesis, cytotoxicity, and microbial resistance.

A full understanding of the ontogeny of immunocompetent cells is required for selective manipulation of the immune system and for correcting imbalances and deficiencies which compromise host immune defenses. For this reason definition of the origin, differentiative pathways, and functional roles of the various subpopulations of lymphocytes continues to be a focus of considerable investigative effort. Although it is certain that one population of mature lymphocytes (T) is thymus dependent and another population (B) is thymus independent, their progenitors and their mechanisms of functional maturation are still not precisely identified. Studies of T and B cell lineage have been greatly facilitated by the recognition that certain lymphocyte surface molecules are characteristically expresssed at specific stages of differentiation and maturation and by the availability of specific antisera to these marker antigens. Dr. Joan Abbott (AI 13759, Sloan-Kettering Institute for Cancer Research) is studying expression of surface markers to define pathways of murine B cell differentiation. She has found that B cells of newborn mice exhibit surface IgM and the Ia gene product of the MHC but lack surface IgD and complement receptor (CR) while B cells from adult mice exibit all four markers. When B cells of newborn animals are treated with lipopolysaccharide (LPS) they promptly acquire the IgD and CR markers. She believes the acquisition of IgD and CR reflects an ontogenic progenitor-successor relationship. Also of interest is her finding that B cells from adult mice promptly lose IgD when treated with LPS but retain the CR marker;

she believes that the loss of IgD under these circumstances reflects another differentiation which is an early event in the development of mature antibody-producing plasma cells.

Dr. Ulrich Hammerling (AI 14831, Sloan-Kettering Institute for Cancer Research) is employing monoclonal antibodies, produced by hybridoma technology, to further define the association of the IgD and CR markers. His data indicate that IgD and CR have a close, spatial, relationship on B cell surfaces. He has found that treatment of B cells with monoclonal anti-IgD markedly reduces the activity of their CR; treatment with monoclonal anti-IgM had no effect on CR activity. Dr. Michael R. Loken (AI 14872, University of Chicago), a recipient of a Research Career Development Award (AI 00348), also is developing monoclonal antibodies by hybridoma technology for study of murine B cell differentiation. Three such antibodies have been partially characterized. One identifies both lymphoid and myeloid cells in bone marrow and is believed to recognize a differentiation surface marker which is lost when cells migrate from the marrow. A second antibody identifies all myeloid cells in bone marrow and detects a very early differentiation marker. The third antibody recognizes B cells in all stages, from precursors to mature cells. These and related monoclonal antibodies to differentiation antigens are now being employed to investigate mechanisms regulating normal B cell differentiation and the abberant differentiation pathways of malignant B cells.

The CBA-N mouse has proven to be a valuable model for study of B cell differentiation. It exhibits an X-linked immune defect in the B lymphocyte population which is believed to result from an arrest of B cell maturation. CBA-N mice lack adult-type IgD-bearing B cells and fail to generate an antibody response to a variety of antigens. Dr. Brigette T. Huber (AI 14910, Tufts University) has demonstrated that the Lyb 3 cell surface marker also is absent in these animals. Because her results indicate that the Lyb 3 molecule functions as a triggering receptor on immunocompetent B cells, she believes the absence of this marker on B cells of CBA-N mice is functionally significant. In related studies, Dr. John M. Fidler (AI 15226, Scripps Clinic and Research Foundation) has demonstrated that the defect in B cell maturation is not absolute but is age associated. He has found that immune responsiveness in these animals improves with age and suggests that their immune deficit reflects delayed, rather than prematurely terminated, B cell development. He also has demonstrated with appropriate cell transfer experiments that their unresponsiveness does not result from suppressor T cell action.

Dr. Alexander R. Lawton, III (AI 11502, University of Alabama) is studying B lymphocyte development in humans to gain insight into the defects in development and function of B cells which occur in immunodeficiency diseases, autoimmune diseases, and lymphoid malignancies. He has demonstrated that one form of congenital agammaglobulinemia is associated with a defect in differentiation of B lymphocytes from their pre-B cell progenitors while failure of pre-B cell development occurs in a type of hypogammaglobulinemia associated with a tumor of the thymus gland. He now has developed a combined immunofluorescent-autoradiographic technique which permits simultaneous visualization of IgA, IgM, and IgD on human B cells. With this technique, he has found that practically all B cells from healthy neonates exhibit all three markers on their cell surface while no such cells were observed in healthy adults. Patients with various forms of congenital agammaglobulinemia are now being examined by this method in an effort to identify defects in B cell differentiation.

The appearance of surface markers on murine T lymphocytes during differentiation also has provided an approach to study T cell lineage. The products of the Tla region, which is closely associated with the murine MHC, have been demonstrated to govern the expression of surface antigens predominantly on differentiated T cells. Dr. Lorraine A. Flaherty (AI 12603, New York State Department of Health) has demonstrated that the Qa antigens, products of a gene within the Tla region, can serve as useful surface markers for identifying immunocompetent lymphocyte subsets. By appropriate breeding experiments, she has identified a series of Qa antigens, each of which appears to define functionally distinct T cell subsets. Preliminary evidence indicates that some Qa antigens are associated with helper and suppressor immunoregulatory functions.

Studies on the ontogeny of lymphocytes being conducted by Irving Goldschneider (AI 09649 and AI 14743, University of Connecticut Health Center) are centered on the identification and isolation of lymphohemopoietic stem and progenitor cells from rat bone marrow. Using specific antilymphocyte sera and the fluorescence-activated cell sorter, he has demonstrated that the Thy-1 antigen, a T cell surface molecule, is present on the least mature members of the B cell series but is absent on mature B cells; for this reason, the Thy-1 antigen can serve as a useful marker of the early stages of B cell development. He also has shown that a subset of bone marrow cells which contain the enzyme terminal deoxynucleotidyl transferase (TdT) is composed almost exclusively of thymocyte progenitors. Supporting evidence for this observation has been obtained recently from the demonstration that a brief exposure of TdT-positive cells to thymosin, a putative thymic hormone, induces expression of the Ly cell surface antigens which are characteristic of functionally mature, differentiated, T cell subpopulations.

Although the functional distinction of lymphocyte populations is convincingly documented, the purpose and mechanisms of their interactions with each other and with macrophages is still being examined. It is clear that B lymphocytes differentiate to become antibody-secreting plasma cells and that differentiated T lymphocyte subsets can exert helper or suppressor effects on B lymphocytes as well as effector functions in transplantation reactions and in cell-mediated immune reactions to various microbial agents. The cooperative and effector functions of T lymphocytes have been found to correlate well with the presence of the cell surface Ly antigen. It also is known that these functions are controlled and regulated by other cell surface antigens which are products of the genes in the MHC. The regulatory role of MHC products and the functional role of other cell surface antigens in the immune response have been defined most extensively in the mouse although systems analogous to the H-2 component of the murine MHC have been identified in other animals and in man.

On recognition of the regulatory role which the MHC exerts on the immune system, efforts to clarify the responsible mechanisms have intensified. To facilitate research in this area, the program has contractually supported the acquistion and distribution of antisera with specificities against various mouse cell surface antigens. A supply of antiserum against many of the H-2 gene products has been made available for distribution to investigators through a contract awarded to Chella S. David (AI 8-2564, Mayo Foundation). Similarly, antisera specific for antigens of the I region of the H-2 complex have been prepared by Donald C. Shreffler (AI 6-2502, Washington University) and are available for distribution to investigators.

Evidence obtained in several experimental systems has convincingly demonstrated that efficient physiological interactions among macrophages, T cells, and B cells require that these cells share membrane molecules encoded for by the MHC of the species. Dr. David H. Katz (AI 13874, Scripps Clinic and Research Foundation) has provided substantial evidence that the genes controlling interactions between T and B lymphocytes are located in the I region of the mouse H-2 complex. He has found that T lymphocytes will not exert effective helper functions for B cells when these cells differ at the relevant I region locus. He believes that self-recognition is the critical mechanism by which cell-cell communication takes place in the immune system. In a related project (AI 13781), he has obtained evidence indicating that the process of differentiation of stem cells and their progeny also may be critically regulated by MHC gene products. Using bone marrow chimeras, he has demonstrated that lymphocyte differentiation may be "adaptive" to the environment in which it takes place. He believes such an "adaptive differentation" results in preferential interactions among cells that have undergone their differentiative processes in the same genotypic environment; "adaptive differentiation" thus may represent a manipulative approach to define genetic and molecular mechanisms responsible for cell-cell interactions.

Genetic restrictions imposed by products of the I region of the H-2 complex on interactions between macrophages and immune T cells are being investigated by Carl W. Pierce (AI 13915, Jewish Hospital of St. Louis). Using antigen pulsed macrophages, he has found that naive lymphocytes will respond to antigen even if it is presented on a histoincompatible macrophage. However, the secondary immune response is genetically restricted; the immure T cell must be stimulated with antigen-bearing macrophages which are genetically identical to the macrophage that presented the antigen during the primary immunization. He has demonstrated that such genetic restrictions are controlled by products of the I-A subregion of the H-2 complex and believes that these restrictions involve active suppressive phenomena when antigen is presented on an inappropriate macrophage.

In a related study, Judith A. Kapp-Pierce (AI 13987, Jewish Hospital of St. Louis) has found that an antigen specific suppressor T cell factor is activated when nonresponder lymphocytes are stimulated by antigen; suppression by this factor is mediated by a molecule encoded by the I-J subregion of the H-2 complex. She also has shown that this suppressor factor does not require participation of macrophages for effective suppression; she suggests that the suppressor factor acts directly on primed B cells. The results of similar studies by Donal B. Murphy (AI 14349, Yale University) suggest that the different functional subpopulations of immunocompetent lymphocytes express different products of the I region on their surface. He has demonstrated that suppressor T cells bear an I region antigen not found on helper T lymphocytes.

Of interest is his observation that, although different T cell subpopulations bear distinct I region products, all or many of these products appear to be expressed on the surface of the macrophage so that its interaction with these lymphocytes would not be encumbered by this type of genetic restriction.

Cell-cell interactions are required not only to initiate the immune response but also to regulate it. It is now recognized that the help and suppression provided by T cell subsets is carefully balanced within the responding host. As demonstrated by Richard K. Gershon (AI 10497, Yale University), helper T cells, on stimulating B cells to produce antibody, also become susceptible to regulation through a feedback inhibition mechanism by inducing suppressor T cells. In collaboration with Harvey Cantor (AI 13600, Sidney Farber Cancer Institute), the functional subsets have been identified on the basis of the Ly differentiation antigens present on their surfaces. Thus, helper cells have been shown to be Ly 1+ while suppressor cells are Ly 2, 3+. Dr. Cantor recently has provided convincing evidence that the Ly 1 helper cell can induce an uncommitted Ly 1, 2, 3+ subset to participate in specific suppressor activity. In this context, Dr. Gershon believes from his current studies on the cellular feedback inhibition mechanism that suppressor dysfunction is a cause of at least some autoimmune diseases. Dr. Cantor has demonstrated recently that feedback inhibition is induced by Ly 1+ cells which also express the Qa 1 cell surface molecule and that selective stimulation of Ly 1+ cells which lack the Qa 1 antigen can negate feedback inhibition mechanisms.

Functionally distinct T cell subsets have also been identified in humans using monoclonal antibodies prepared against human thymocytes. Evidence is accumulating that the T4 antigen is present on a mature T cell subset in the peripheral blood population which has helper function; reciprocally, the presence of the T5 cell surface antigen has been correlated with the suppressor population of peripheral T cells. Dr. Cornelius P. Terhorst (AI 15066, Sidney Farber Cancer Institute) has provided physicochemical evidence that the T4 and T5 antigens are the human homologs, respectively, of the murine Ly 1 and Ly 2, 3 antigens. He has found that the T4 and T5 antigens can be isolated using antisera against murine Ly antigens and that the isolated human antigens have molecular size and structural features comparable to those of murine T cells.

Although research on functional subsets of T lymphocytes is proceeding at an accelerated pace, it is recognized that this work is technically limited by the need to separate the subsets in adequate numbers for study. This difficulty may be obviated by the recent work of Frank W. Fitch (AI 04197, University of Chicago) who has developed a method to isolate and culture cloned lines of reactive T cells. Two types of T cell clones have been derived. One is cytolytic and expresses Ly 2 alloantigens; the other is non-cytolytic, expresses Ly 1 antigen, and exhibits helper functions. The latter, in mixed culture, can promote proliferation of cytotoxic cell clones and, in isolated culture, generates a soluble factor which can activate cytotoxic cell clones. Another approach is that being explored by Fritz H. Bach (AI 15486, University of Wisconsin) who is attempting to produce T cell hybrids by fusing immune mouse T cells with mouse T lymphoma cells. Preliminary results are encouraging; the hybrids express the Thy-1 antigen of normal lymphocytes and produce soluble factors which suppress cytotoxic cell activation.

The development and improvement of technology for T cell cloning can be expected to markedly facilitate studies of cell-cell interactions and of immunoregulatory mechanisms in the future. For the same purpose, the Program's contractual efforts are being concentrated on providing useful research reagents. The acquisition and distribution of Ly antisera and the preparation of antisera against newly-recognized Ly antigens and other immunologically-relevant cell surface molecules are being supported by a contract with Edward A. Boyse (AI 8-2541, Sloan-Kettering Institute for Cancer Research). The recent award of a contract to Anne W. Hamburger (AI 0-2655, American Type Culture Collection) for the acquisition, characterization, storage and distribution of hybridoma cell lines also is intended to support and facilitate investigator-initiated research studies of the immune system.

3. Immunochemistry

Research grouped in this program category centers on the defined molecular components of the immune system and utilizes chemical and physicochemical approaches for study of immunologic reactants and reactions. Studies on the chemical composition and structure of humoral and secretory immunoglobulins and of natural and synthetic antigens, as well as of the mechanisms and kinetics of antigen-antibody reactions and of the mechanisms of immunoglobulin biosynthesis and its regulation, are included within this program area. Also relevant are studies at the molecular level of cell surface components related to immunologic activity and of subsurface cytoskeletal and contractile assemblies believed to play a role in cellular activation. In addition, the newly-developing field of immunopharmacology, which is concerned with the identification, characterization, isolation, or synthesis of chemical regulators of immune function and the elucidation of their mechanisms of action, is supported in this program area.

A major aim of studies supported by this program is to elucidate the primary structure of antibodies and to correlate their structure with their antigen binding, specificity, and other biological functions. The results of structural studies have established that an immunoglobulin basically consists of two identical light (L) and two identical heavy (H) chains, linked by disulfide bridges to form a two-fold symmetrical molecule, each side of which consists of an L and an H chain. Two classes of L chains (kappa and lambda) and five H chains (gamma, alpha, mu, epsilon and delta), which determine the immunoglobulin class (IgG, IgA, IgM, IgE, and IgD, respectively) have been recognized. Limited proteolysis of a typical IgG molecule results in three fragments of nearly equal size, 2 Fab fragments and an Fc piece. It is now clear that that Fab components contain the antigen binding (combining) site and that the Fc fragment plays an important role in complement fixation. Amino acid sequence studies of immunoglobulins have demonstrated the existence of three domains with constant sequences on the heavy chain (C_H) and one domain of variable sequence (V_H); each light chain consists of two domains, a constant region (C_L) and a variable domain (V_L). The close association of the V_L and V_H domains in the Fab fragment, containing the combining site, produces a continuous hypervariable surface which can be readily altered by amino acid substitution, insertions or deletions and provides an extremely large number of structural combinations which are believed to confer immunologic specificity.

Considerable effort now is being expended to gain an understanding of the mechanisms controlling and regulating immunoglobulin biosynthesis and of the basis for antibody diversity. Attempts to explain the diversity of variable region sequences have centered on a multiple germ-line gene theory which presupposes that all information necessary for antibody production is encoded in the germ line and is inherited or on a theory of somatic diversification which assumes that a limited number of germ-line genes are diversified during an individual's lifetime by somatic processes, such as point mutations, recombinations or introduction of errors into the gene sequence, followed by repair to yield a new set of encoding genes.

Amino acid sequence analysis and structural studies provide one approach to understanding immunoglobulin diversity. A contract project under the direction of Elvin A. Kabat (AI 8-2158, Columbia University) supports the tabulation and analysis of immunoglobulin sequence data reported in the world's scientific literature. Using the PROPHET Computer System, this project provides the concerned scientific community with a unique and invaluable data resource and serves as a source of data for molecular model building and statistical predictions of immunoglobulin molecular structures. The accumulated data base on immunoglobulin variable region sequences was first published in September 1976 and a second, expanded, edition appeared in October 1979. The recent publication contains 26,108 sequenced amino acid residues of L chains and 11,147 of heavy chain V regions, as compared, respectively, with 16,118 and 7,516 amino acid residues in the first edition. A series of computer-assisted analyses of the data base has been completed; evidence has been obtained to demonstrate that the amino acid sequence characteristic of segments in the variable region of an immunoglobulin is uniquely associated with antibody specificity. Computer-generated inferrences concerning amino acids at key positions in light and heavy chains agreed well with structural conclusions obtained by X-ray crystallography. Additional predictions of the three dimensional structure of the combining site have supported the suggestion that the structural gene for the variable region of an antibody may be assembled from "mini genes" which have been conserved in the germline. Support for the germline theory also has been provided from research studies conducted by Joseph M. Davie (AI 11635, Washington University) and Fred Karush (AI 09492, University of Pennsylvania).

However, using antibodies prepared against the combining site, or idiotype, of an immunoglobulin, Alfred Nisonoff (AI 12895, Brandeis University) believes that it is not necessary for a mouse to inherit V_H structural genes. He feels that antibody diversity can be generated through a random process of somatic mutation, rather than through a programmed, genetically-controlled series of mutations. The results of research by Matthew D. Scharff (AI 05231, Albert Einstein College of Medicine) and Malcolm L. Gefter (AI 13357, Massachusetts Institute of Technology) also support the view that immunoglobulin diversity is generated by somatic diversification. Furthermore, the results of sequence studies of DNA and messenger RNA and of their function in immunoglobulin synthesis conducted by several investigators including Randolph Wall (AI 13410, University of California at Los Angeles), and Janet M. Stavnezer (AI 14617, Sloan-Kettering Institute for Cancer Research) are difficult to reconcile with a strict germ-line theory and argue for a somatic process of immunoglobulin diversification. Based on amino acid and DNA sequence studies, Leroy E. Hood (AI 10781, California Institute of Technology) has proposed the existence of a new gene segment, D, between the V and J segments which by combinatorial joining of multiple V, D, and J segments may result in considerable antibody diversity.

Although sequence and serologic studies of serum immunoglobulins are not generally restricted by the availability of material for study, similar studies of cell-bound immunoglobulins and other cell surface receptor molecules have indeed been limited by the small amounts of material available for study. Recent technical advances may obviate this problem, however. Dr. Gerald M. Edelman (AI 09273, Rockefeller University) had developed a system to synthesize authentic mouse beta-2 microglobulin and its precursor in rabbit reticulocytes using messenger RNA from murine tumor cells; its authenticity has been verified by radiochemical-sequence analysis. The same system is now being used to synthesize H-2 antigens, TL antigens, and other cell surface proteins found on lymphoid cells. Similarly, Sheldon Dray (AI 04073, University of Illinois Medical Center) has been employing liposomes to selectively insert functional messenger RNA into cultured cells. He has obtained evidence which indicates that cultured human epithelial carcinoma cells treated with liposomally-encapsulated rabbit globin messenger RNA are stimulated to produce a globin-like protein. Dr. Matthew D. Scharff (AI 05231, Albert Einstein College of Medicine) is employing somatic cell genetic technology to refine techniques for production of hybridomas. He has developed an extensive library of drug-marked myeloma cells for use in fusion experiments and has adapted the fusion technology to significantly improve the frequency of functional hybridomas. Dr. John F. Kearney (AI 14782, University of Alabama in Birmingham), a recipient of a Research Career Development Award (AI 00338) has been particularly and uniquely successful in applying hybridoma technology to study early immunoglobulin gene expression in the first identifiable cells of the B cell lineage. His pre-B cell hybridomas are providing individual light and heavy chains of IgM in sufficient quantity for physicochemical and sequence studies. Further application of the hybridoma technology, as well as the insertion and translation of messenger RNA in cultured cells, has the potential to provide an unlimited source of homogeneous antibody and other immunologically-important molecules for study. Application of recombinant DNA technology to this problem can similarly be expected to expand the supply of study material. Randolph Wall (AI 13410, University of California at Los Angeles) already has successfully inserted mouse myeloma immunoglobulin DNA into a bacterial plasmid and has isolated recombinant clones which contained all of the light chain constant and variable region coding sequences.

Efforts to define and understand the molecular basis of antigenicity and of the antigenic or determinant site similarly have progressed by application of amino acid sequence analysis. For example, M. Zouhair Atassi (AI 13181, Mayo Foundation) and Ahmed F. Habeeb (AI 14791, University of Puerto Rico) have employed hen egg white lysozyme as a model antigen and have systematically sequenced it. They have identified three peptide fragments which contain the antigenic sites of this protein. Using a method of surface simulation, they have synthesized peptides containing the component amino acid residues in appropriate structural order and have been able to accurately define the boundaries, residues, and conformational structure of the three determinants of the native protein. Their results clearly demonstrated that these three sites quantitatively accounted for the total antigenic reactivity of the native protein. Thus, the entire antigenic structure of lysozyme has been precisely defined. In a related study, Eli E. Sercarz (AI 11183, University of California at Los Angeles) has demonstrated that peptide fragments containing these determinants can be distinguished by their different immunologic functions. The L_1 peptide, containing amino acid residues 1-12, induced a suppressor cell response; the L_2 peptide, containing amino acids 13-105 induced helper cells while the L_3 peptide had no effect. This demonstration of helper and suppressor sites on the same molecule provides a powerful tool to investigate the cellular mechanisms of immune recognition.

MICROBIOLOGY AND INFECTIOUS DISEASES PROGRAM

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MICROBIOLOGY AND INFECTIOUS DISEASES PROGRAM

Annual Report, 1979-1980

Director's Report

Introduction

The accomplishments of the five branches of the Microbiology and Infectious Diseases Program (MIDP) are summarized in the following sections; activities in International Health are reviewed at the end of this section. Selected highlights are summarized below.

Previous annual reports have emphasized that infectious diseases are still responsible for nearly 25% of all visits to physicians in the U.S. each year, and account for 65% of all such visits for children under 16 years of age. While many of these are mild illnesses in otherwise healthy individuals and are responsible for only temporary absence from work or school, it is emphasized that infections can be serious, even fatal, particularly in certain groups of individuals such as the elderly and those with underlying chronic disease. For example, a recent international symposium noted that infections are now the leading cause of death in patients with cancer; the cytotoxic drugs administered in the hope of killing cancer cells also damage the bone marrow and other components of the immune system. Thus, a major challenge in the management of this expanding group of patients is how best to prevent and to treat bacterial and mycotic infections.

Apart from mortality, infections are an important cause of morbidity in individuals who are at risk because of other underlying conditions or simply because of advancing age. A recent study of an approach to health care delivery in the inner city (Master, R.J., et.al., New Eng. J. of Med. 302: 1434-1440, 1980) noted that the system cared for 3,638 chronically ill, elderly, home-bound and nursing home residents during a representative year. These individuals were hospitalized at a rate of 119 admissions per 1000 patients per year, with the reasons for hospitalization varying depending on the group. Acute bronchopulmonary conditions (asthma, bronchitis, pneumonia, and pulmonary insufficiency) ranked first as a cause of hospitalization in the nursing home population (mean age 75.0), with other infectious diseases (pyelonephritis, cellulitis, gram-negative sepsis, etc.) ranking fourth both for this group as well as for the home-care population (mean age 79.0 years). In the ambulatory population (mean age 48.8 years), these two problems ranked fifth and sixth as causes of hospitalization. In summary, acute bronchopulmonary conditions and other infectious diseases accounted for 30.4% of hospitalizations from the nursing home population, for 17.6% from the home care group, and for 13.4% from the ambulatory patients. Nationwide, the costs of such infections are enormous both in terms of ill health and in terms of expenditures for hospital care and antibiotics. It is estimated that infections secondary to indwelling catheters in nursing home and hospitalized patients account for more than 30% of nosocomial infections. Each medical advance exacts its price. All too often, it is the ubiquitous microbes that inhabit us and/or surround us that are responsible. The new heart valve or new hip that have been replaced by skilled surgeons may become infected by bacteria resistant to antibiotics. The technically successful renal transplant may fail because of activation of a cytomegalovirus (CMV) infection. The normal young infant may die or be mentally retarded for life because it is infected at birth with CMV, a herpes simplex virus, or Group B hemolytic streptococci.

Although medicine's biggest triumph has been the control and virtual elimination of certain infectious diseases in the developed countries of the world, old problems remain and new ones are constantly emerging. Some of these new problems are truly "new", in that they represent the price of progress mentioned above; others are "new" in that they have been identified for the first time, e.g., Lassa fever, Legionnaire's disease, infant botulism, rotavirus diarrhea, and, identified by exclusion, non-A, non-B viral hepatitis. Despite the magnitude of these problems, solutions forecast by current research give reasons for optimism, and it is gratifying to conclude these paragraphs with an optimistic note that records a remarkable achievement. During the year, the World Health Organization declared that smallpox had been eradicated - totally eliminated from the World.

Administration

Sir Charles Stuart-Harris: During the first six months of 1980, MIDP was privileged to share, along with all of NIAID, the talents of Sir Charles Stuart-Harris, Professor of Medicine Emeritus, University of Sheffield, who was completing his appointment as a Fogarty Scholar. Sir Charles is an international authority on influenza, and participated actively in the review and evaluation of the influenza program, including serving as a site visitor to a number of laboratories and vaccine evaluation centers. In collaboration with Drs. David Alling and William Blackwelder he completed an analysis of excess mortality due to influenza, an analysis that emphasized once again the seriousness of epidemic influenza. A second major contribution was the writing of an objective critique of the current status of live influenza virus vaccines. On behalf of the Fogarty International Center, he organized a series of six seminars on various aspects of infection and immunity, and played a major role in organizing a special meeting to consider whether or not there are diseases other than smallpox that may be candidates for worldwide eradication. We are most appreciative of his contributions and hope that he will return often.

Vaccine Development: Work continued on some three dozen possible bacterial, parasitic and viral vaccines. In some instances only one or two grantees were conducting preliminary studies; in other instances, such as with hepatitis B vaccine, extensive field trials were in progress. Under the new bilateral cooperative health agreement with the People's Republic of China, a protocol has been developed for a trial of the vaccine developed by NIAID intramural scientists in the prevention of maternal-infant transmission of hepatitis B.

Another international program led to consideration of expanded vaccine development activities. Vaccine research and development was selected as a component of the expanded U.S.-Japan Non-Energy Research Cooperation, and this subject is to be considered at the sixteenth meeting of the Joint Committee of the U.S.-Japan Cooperative Medical Science Program in October. Scientists in both countries are aware that the newly developed recombinant DNA and hybridoma technologies will greatly facilitate the identification and purification of microbial antigens and accelerate the development and eventual utilization of new vaccines. An initiative to expedite the development of vaccines is timely since investigators are now better able to identify specific microbial antigens and to test their immunogenicity; it is urgent to the extent that bacteria are becoming resistant to antibiotics, e.g., in some U.S. localities, 15% to 33% of strains of Hemophilus influenzae are now resistant to ampicillin.

Consensus Conference: At the beginning of the year, October 15-16, 1979, MIDP sponsored a Consensus Development Conference on the role of amantadine in the prevention and treatment of influenza. Coordinated by Dr. John LaMontagne and chaired by Dr. Jay P. Sanford, the meeting was considered highly successful, the conference summary and recommendations being given wide publicity. A study is now in progress to evaluate the impact of the conference on the prescribing practices of U.S. physicians.

Reye's Syndrome: As a consequence of MIDP initiative in reviewing Reye's Syndrome during a workshop on influenza B virus, NIAID has been assigned the lead role in coordinating trans-NIH research in this puzzling, serious disease. A consensus conference on the subject is being planned by a committee chaired by Dr. John LaMontagne.

Epidemiology and Biometry Branch: During the year the enteric diseases program was transferred to the Epidemiology and Biometry Branch following the departure of Dr. Carl Miller from the Development and Applications Branch. Dr. George Curlin, Chief of EBB, who has extensive experience in diarrheal diseases, also served as Enteric Diseases Program Officer and as Executive Secretary of the Cholera Panel, U.S.-Japan Cooperative Medical Science Program.

The new PHS epidemiology training program was initiated during the year, and NIAID was able to compete successfully for one of the five (of a total 12) Epidemiology Associate Positions assigned to NIH. Dr. Alfred Saah, a former EIS officer and internist with training in infectious diseases began his appointment in July.

Molecular Biology and Recombinant DNA

Microbiology occupies a central role in the development of both cell and molecular biology. Indeed, as noted by W.K. Joklik in his presidential address to medical school microbiology chairman, "microbiology spawned molecular biology", making it possible to investigate the structure and function of nucleic acids and proteins using not only biophysical and biochemical, but also genetic techniques.

Studies of plasmid mediated antibiotic resistance are addressing the very practical problem of therapy by seeking to understand the mechanisms involved in the development of drug resistance while at the same time using transposable drug resistance elements as tools in the genetic analysis of bacteria. Mapping of bacterial genes, in turn, will lead both to a greater understanding of why virulent organisms are pathogens and to more precise gene splicing in avirulent ones, i.e., low risk recombinant DNA experiments.

Studies of the molecular biology and genetics of bacterial plasmids led to the technological breakthrough the made it possible to recombine genetic material so as to construct DNA molecules programmed to direct the synthesis of biologically useful substances such as hormones, antibiotics, interferon, and viral antigens. Thus did basic research spawn a whole new industry — bio-or genetic engineering — an industry that expanded rapidly during the past year as investigators and investors sought to exploit this new technology.

Advances in antiviral chemotherapy are anticipated based on the use of genetic cloning techniques to isolate large amounts of protein coded by viral genomes. This will facilitate study of the structure and function of these proteins and the design of inhibitors specifically constructed to inhibit their functions. Coupled with this is the need for rapid viral diagnosis and the expectation that hybridoma produced monoclonal antibodies directed at the type-specific components of viruses will permit prompt identification of minute amounts of viral antigens in body secretions, fluids, and tissues.

Already, the double stranded DNA of hepatitis B virus has been cloned in *E. coli* K12 by several groups, with the hope that the bacterium will synthesize viral surface antigens that can be used, instead of viral particles from plasma, in the production of a hepatitis B vaccine. Other investigators have used this approach to synthesize the hemagglutinin antigen of influenza virus for use in inactivated influenza vaccine. Still others have reported the extension of this technology to the cloning of genes in mammalian cells (e.g., to synthesize hepatitis B viral antigen in tissue culture) a step which, it is suggested, will make it possible to cure genetically determined inborn errors of metabolism at the molecular level by replacing defective genes with normal ones.

Diarrheal Diseases

There are certain questions that can best be answered, sometimes only answered, by studies in volunteers. Two of the studies conducted at the Vaccine Development Center at the University of Maryland during 1979-1980 are illustrative.

Escherichia coli: A purified, safe *E. coli* pili type 1 vaccine injected intramuscularly was found to significantly protect volunteers against diarrhea, malaise and vomiting induced by oral challenge with virulent, enterotoxigenic *E. coli*. This provides direct evidence that bacterial type 1 pilus is a virulence factor in man, and offers hope that pilus vaccines can be developed in the future against *E. coli*-induced traveler's diarrhea.

Vibrio Cholerae: Volunteers challenged two to three years earlier with classical Ogawa *V. cholerae* were fully protected against rechallenge by the same organism. These results provide the first definitive evidence in man that cholera immunity produced by a "natural" cholera infection is relatively long-lasting, and encourages further cholera vaccine development efforts.

Sexually Transmitted Diseases (STD)

During the past two decades, it has been recognized that a number of agents in addition to those of the five classical venereal diseases (gonorrhea, syphilis, chancroid, lymphogranuloma venereum, and donovanosis) are sexually transmitted. These "new" STD agents include *Chlamydia trachomatis*, *Mycoplasma hominis*, herpes simplex viruses, CMV, *hepatitis B virus*, and *Group B streptococci*. During this same time, an increase in the population of young adults plus changes in sexual behavior and in contraceptive practices led to a dramatic increase in the incidence of all STD.

In January, 1980, NIAID asked a Study Group of nine panels, coordinated by Dr. Milton Puziss and chaired by Dr. King Holmes, to review current progress and to make recommendations as to research directions and needs. The report and recommendations of the STD Study Group were endorsed by the Institute's Advisory Council in May, 1980, after modifications in the section on training and in suggested funding levels. The STD summary report might well be regarded as an appendix to this Annual Report.

Bacteriology

Gonococci: One episode of pelvic inflammatory disease confers immunity to a second type of salpingitis due to the same POMP (Principal Outer Membrane Protein) type of gonococcus. This is cited as the first example of immunity following a naturally occurring gonococcal infection. Guinea pigs immunized with POMP antigen are protected against infection. In addition to antibodies to the POMP antigen, opsonic antibodies are directed primarily against the antigens of the pili, the flagellar-like organelles of attachment. Both POMP and pili antigens have been purified and incorporated into experimental vaccines now undergoing early Phase I trials in humans.

Group B Streptococci: Progress in the isolation and characterization of the polysaccharide antigens of types Ia, Ib, Ic, II and III is being made by two groups of investigators. Several antigens are ready for early trials in volunteers.

Mycobacteria: Although the tuberculin test has been a very useful diagnostic and epidemiological procedure, a continuing problem in the U.S. has been that of specificity because individuals may have been infected with one of the numerous antigenically cross-reacting mycobacterial species present in the environment. Daniel and associates at Case Western University have now reported the isolation of an antigen that by immunodiffusion and immunoelectrophoresis appears unique to *M. tuberculosis* and *M. bovis*. If this is so, it will now be possible to produce a specific skin test antigen, one that will be particularly valuable in those parts of the world where tuberculosis is still a major problem. Considering the time that it has taken to reach this point with *Mycobacterium tuberculosis*, progress with the as yet uncultivatable *M. leprae* has been remarkable. One investigator (Buchanan, U.S. Public Health Service Hospital, Seattle) has identified a protein antigen that appears to be specific for *M. leprae*, while another investigator (Brennan, National Jewish Hospital, Denver) has purified a lipid which appears to be a species specific antigen. Should these findings be confirmed, the availability of species specific antigens will greatly facilitate epidemiological studies of leprosy and provide a basis for the approach to immunization undertaken by the World Health Organization.

Parasitology

Biologic Control of Vectors: Among studies in this challenging field, the following gems are worthy of note:

- A nematode that kills Simulium black flies, the vector of onchocerciasis.
- A gregarine protozoan that kills *Aedes triseriatus* mosquitoes, the vector of California and LaCrosse encephalitis viruses.
- A large non-vector mosquito that competes with vector *Aedes* mosquitoes in nature.
- An anti-feedant extracted from the bark of an African tree that irreversibly turns off an insect's sense of taste, and, as a bonus, kills the snail vector of schistosomiasis.

Malaria Vaccine: Monospecific anti-sporozoite antibody produced by monoclonal hybridoma cells (successfully employed with *Plasmodium berghei*, the parasite of rodent malaria) is being used to isolate and purify the protective antigens of the sporozoites of *P. falciparum*, the most virulent form of human malaria.

Immunology: Immunologic responsiveness plays a major role in the granuloma formation of schistosomiasis; host defense mechanisms of Chagas' disease are under thymic control; the antifilarial action of diethylcarbamazine may depend on the immune elimination of microfilariae after the drug has stripped the parasite of a protective coat of immune origin.

Virology

Herpes Viruses: Sensitive molecular probes have been used by Huang, University of North Carolina, to detect DNA of CMV and herpes simplex viruses in normal cervixes and in cervical cancers from various geographic regions. The evidence to date is not convincing that either herpes simplex or CMV induce human cervical carcinoma.

The promising new antiherpes drug, acyclovir, is dependent for its action on the induction of a virus-specified thymidine kinase (TK). Perhaps an indication that viruses will be just as adaptable as bacteria is the demonstration that two mutants resistant to acyclovir induce DNA polymerase activities controlled by a gene other than the one that regulates TK activity.

Rabies Vaccine: The new human diploid cell culture rabies vaccine developed by Kaprowski, et.al., of the Wistar Institute, and used with great success abroad, was licensed for use in the U.S. in May. Expectation of continuing success is based on the assumption that rabies viruses isolated throughout the world are antigenically closely related. Now, the same investigators, using monoclonal antibodies that recognize single antigenic determinants, have identified a number of variants. Analysis of reactivity patterns of seven virus isolates from fatal human U.S. cases with a panel of 14 antiglycoprotein monoclonal antibodies separated these strains into four groups. Preliminary results of challenge studies of vaccinated mice recorded only partial cross-protection between groups. These findings have prompted the Pan American Health Organization to initiate studies to evaluate protection between rabies vaccines in a given area and street viruses isolated from animals and humans who died of rabies in the same area.

International Health

International health research programs are administered by Dr. Earl Beck, Special Assistant to the Director, MIDP, in conjunction with individual program officers and coordinators. Dr. Beck was responsible for the design and implementation of a major program change during the past year.

The International Centers for Medical Research Program (ICMR) which had been funded since the early 1960's was phased-out on May 31, 1980. This Program served a very important and useful role in promoting research and training in tropical medicine. Linkages were established at overseas sites that provided opportunities for U.S scientists to study tropical diseases where they occurred. The four institutions and their overseas sites were:

1. The University of California ICMR in San Francisco collaborating with the Institute of Medical Research and the University of Malaya, Kuala Lumpur, Malaysia.

2. The Johns Hopkins University ICMR in Baltimore which completed its research effort at the Gorgas Memorial Laboratories in Panama after working in previous locations in India and Bangladesh.

3. The University of Maryland ICMR in Baltimore and in Lahore, Pakistan, with the Pakistan Medical Research Center.

4. Tulane University in New Orleans collaborating with COLCIENCIAS (The Columbian Fund for Scientific Research; an organization similar to the NSF in the U.S.), in Cali, Colombia.

During the phase-out year, the ICMR's continued to be productive; briefly, the Hopkins group in Panama is working intently to finish some diarrheal disease studies in several Panamanian populations. These include agent isolation, characterization and identification along with epidemiological and oral rehydration studies. The grant was extended three months without funds for the completion of this work. Also, the Tulane group reported as a first the infection of a primate with a Haitian strain of Mansonella ozzardi (filaria); and two new or previously unreported strains of filaria found in southeastern Colombia and in Venezuela are under intensive study to determine their potential as agents of human disease. The California group continued to add to our knowledge about the jungle cycle of dengue, its vectors and their relationship to rural and urban populations. Work was continued by the Maryland group in Pakistan to develop a biological system which will control the vectors that transmit malaria. A genetic sexing system using dieldrin resistant male Anopheles culicifacies was developed; the females that do not carry the resistant gene are killed, facilitating mass production of male mosquitoes. Male mosquitoes are chemo-sterilized and used in field release studies. Preliminary laboratory data are encouraging.

International Collaboration in Infectious Diseases Research (ICIDR), a new initiative that supersedes the previous ICMR program was announced in the May 12, 1978, issue of the Guide to Contracts and Grants. The program is divided into Part A, International Program Project Grants, and Part B, International Exploratory/Developmental Research Grants, to provide increased programmatic flexibility and to insure maximum opportunity for investigator participation and collaboration. The research emphasis of the new ICIDR program is infectious diseases and the immunology of these diseases. Special attention is given to the six diseases of the WHO Special Program for Research and Training in Tropical Diseases (malaria, schistosomiasis, filariasis, trypanosomiasis, leishmaniasis, and leprosy), although any disease of sufficient health importance to a developing country was not excluded. Part A, International Program Project Grants, embraces broadly based multidisciplinary research programs with a well-defined central focus or objective, and with the major portion of the research (80%) being conducted overseas with an acceptable foreign affiliate in a developing country. Proof of a truly collaborative research effort was required before the proposal was considered. Fourteen program project grant proposals were reviewed, 9 were approved. Of these, 4 were funded in September, 1979, and the 5th was only recently funded in June, 1980.

Part B, International Exploratory/Developmental Research Grants, is designed to encourage the individual investigator to develop a biomedical research program with an overseas affiliate, with the major portion of the work performed in the host country. These are planning type grants that should lead to the development of a large program project (P01) or a regular research grant (R01), or a foreign grant (F). Thirty-nine grants were reviewed, 16 were approved, and 5 were funded in FY 1979.

Although all four of the ICMR's submitted grant proposals (P01's), only one, Tulane University, competed successfully for ICIDR support. This was rather unexpected, since the ICMR's already had ongoing programs with an established foreign affiliate and had access to the necessary authorities to obtain official clearances from the host institution and the Ministry of Health or some other high-ranking government official. The clearances proved to be an insurmountable hurdle for some prospective applicants because of the proposal receipt deadline. When this program is readvertized, it is planned to allow at least a year for preparation of proposals with accompanying assurances.

The 10 ICIDR grants have been funded for less than a year, but all of the grantees are tooling-up rapidly, and some have already started their research and collected data. The Program has been well received, with a tremendous amount of enthusiasm generated by the U.S. grantees and their foreign counterparts. At this point, it would appear that the Program has great potential for fulfilling all the stated research objectives plus establishing some firm and enduring collaborative relationships as partnerships in health. The 10 institutions and the U.S. principal investigators participating in the ICIDR Program and their respective areas of research interest are:

Part A (P01) - Program Project Grants

1. Cornell University Medical College, New York City, with Drs. Thomas Jones and Warren Johnson as Co-Principal Investigators. Collaboration is with the Federal University of Bahia and with the University of Brasilia in Brazil, on cutaneous, mucocutaneous, visceral leishmaniasis and trypanosomiasis. The Cornell investigators had previously worked in Brazil so their administrative arrangements with the Universities and the Ministry of Health proceeded nicely. The field areas and populations have been identified and work is in progress.

2. Harvard School of Public Health, Boston, with Dr. Thomas Weller as the U.S. Prinicpal Investigator, is collaborating with the Federal University of Bahia and the Oswaldo Cruz Foundation, Brazil, on Chagas' disease. Dr. Weller and his group had worked in Brazil earlier and had identified field areas and populations for studying various aspects of Chagas' disease. Although the research is progressing nicely, there appears to be some difficulty in establishing a workable financial accounting system.

3. Michigan State University, East Lansing, with Co-Principal Investigators Drs. Jeffrey Williams and James Bennett, is associated with the Central Laboratories, Ministry of Health, Khartoum, Sudan, with the main field area located in Wau in the southern part of the country. The Sudanese Government is very pleased to have this group associated with its well-trained scientists, hoping to reduce the brain-drain to affluent neighboring countries. The Ministry of Health has contributed $40,000 toward renovations of laboratories and animal quarters. The country is exceedingly poor, the inflation rate is high, the black market is flourishing, and the amount of research material abundant. Dr. Bennett and his family have been in Khartoum since January, 1980. The investigations will concentrate on malaria, schistosomiasis and onchocerciasis. This is a good group with a great deal of enthusiasm and perserverance and is guided by well-qualified and experienced

investigators.

4. Tulane University, New Orleans, with Dr. Thomas Orihel as Principal Investigator is collaborating with COLCIENCIAS in Cali, Colombia. This was the only ICMR to compete successfully for an ICIDR grant. The collaborative effort will be concentrated on filariasis, trypanosomiasis, and onchocerciasis. Funding of this project was delayed until June 1, 1980, since it had already been awarded support as an ICMR to that date.

5. University of Illinois, with Drs. Kenrad Nelson and Victoria Schauf as Co-Principal Investigators, is collaborating with scientists at Chiang Mai University and McKean Rehabilitation Institute, Chiang Mai, Thailand. The collaborative effort will be concentrated on various aspects of leprosy in designated field study areas. The laboratories are operative and field studies are in progress.

Part B (R-21) Exploratory/Developmental Grants

1. The University of Hawaii, with Dr. Wasim Siddiqui as the Principal Investigator, is collaborating with his counterpart in Aligarh Muslim University, India. Work is in progress to cultivate human malarial parasites in vitro, and it is proceeding well. Each time Dr. Siddiqui visits Aligarh he attracts scientists from other institutions some distances away to observe and to discuss malaria research. This is one example of how it was hoped that the ICIDR program would promote research training and institute strengthening.

2. Columbia University, New York, with Dr. Philip D'Alesandro as the Principal Investigator, is collaborating with scientists at the Federal University of Rio de Janeiro, Brazil. The research is directed at characterizing surface receptors and studying immunoregulatory mechanisms of visceral leishmaniasis.

3. University of South Florida, Tampa, with Dr. Gary Rodrick as the Principal Investigator, is collaborating with scientists at Fundacao Oswaldo Cruz, Brazil. The reports from Dr. Rodrick are enthusiastic and highly laudatory for Dr. Paraense and his wife, who are experts on molluscs. One of the aims was to determine whether Biomphalaria straminea (B. straminea) could possibly act as a vector for Schistosomiasis mansoni. Laboratory and field studies confirmed that B. straminea is, indeed, a vector. The importance of such a finding can be appreciated when it is known that the Brazilian population is moving into the hinterlands where these snails are plentiful. Dr. Rodrick can say only good things about the Oswaldo Cruz and the opportunities for exchange with scientists from other countries who are visiting and working at the Foundation.

4. Ohio State University, Columbus, with Dr. John Briggs as Principal Investigator, is collaborating with scientists at the University of Nigeria, Nsukka. Dr. Briggs and his Nigerian collaborators are investigating biological control as a means of controlling vectors of human disease. In Dr. Briggs' last report he was concerned with the dearth of trained scientists.

He felt that the key to success was to concentrate on the pre- and postdoctoral students.

5. University of Washington, Seattle, with Dr. Thomas Buchanan as the Principal Investigator, is working closely with scientists at the National School of Biological Sciences, The National Polytechnic Institute, Mexico City. The investigators are studying the serodiagnosis of subclinical leprosy using Buchanan's surface antigens and comparing the results with Dr. Abe's method. Also, there will be an attempt to determine whether there are strain differences among Mycobacterium leprae.

The U.S.-Japan Cooperative Medical Science Program has been operative since 1965. The program provides the basis for collaborative research between scientists of the two countries on diseases of importance to Asia, although some are common to the United States and Japan as well as worldwide. For each disease or disease category a panel of scientific experts is appointed in each country to provide scientific advice and guidance. At present there are panels for the following: cholera and cholera-like diseases, environmental mutagenesis and carcinogenesis, hepatitis (viral), leprosy, malnutrition, parasitic diseases (schistosomiasis and filariasis), tuberculosis and viral diseases (arboviruses, rabies, rotaviruses).

There is considerable enthusiasm for adding a panel on immunology that would be the first discipline-oriented panel to be added since the beginning of the Program. An immunology Symposium is to be convened the three days preceding the Joint Committee meeting in Tokyo in October, 1980. It is anticipated that the formation of this panel will be formally announced at the Joint Committee meeting pending the submission of acceptable panel guidelines.

The annual meeting of the U.S. Delegation was held at the State Department on June 23, 1980, to prepare for the Joint Committee meetint to be held on October 16 and 17, 1980 in Tokyo. Items discussed included U.S.-Japan Non-Energy Research and Development Cooperation, internationalization of the Leprosy and Parasitic Diseases Panels, and the eventual phase-out of the Tuberculosis Panel. These will be agenda items for joint discussion at the October Committee Meeting.

U.S.-U.S.S.R Health Cooperation: In accordance with the U.S.-U.S.S.R. Collaborative Agreement on Influenza and Acute Respiratory Diseases, the first meeting and Symposium on Immunoprophylaxis of Influenza was held in Middletown, Virginia, on October 16-18, 1979. Ten Soviet and 12 U.S. scientists participated.

U.S.-P.R.C. Cooperation in the Science and Technology of Medicine and Public Health. For the period May 17-23, 1980, NIAID was host to Drs. Huang Zhen Xiang and Li Yiu Lin, Vice Directors of the Institute of Virology, Chinese Academy of Medical Science, Beijing. Dr. Huang is the Chinese Coordinator for Infectious Diseases (U.S. Coordinator is Dr. William H. Foege, Director, CDC), and was visiting U.S. Laboratories to explore potential collaboration in influenza and hepatitis research. The Chinese have assigned priority to hepatitis. A protocol for a collaborative study of hepatitis vaccine was drafted by Dr. Huang and Drs. James Maynard (CDC) and Robert Purcell (NIAID).

BACTERIOLOGY AND VIROLOGY BRANCH

The Bacteriology and Virology Branch is responsible for the administration of a broad-ranging program of biomedical research; it supports program project grants, individual research grants, training grants, career development awards, individual postdoctoral fellowships, and contract programs of targeted research in bacteriology, virology, and mycology. Dr. Milton Puziss administers the Branch and the Bacteriology Program. Dr. William P. Allen administers the Virology Program, and also serves as Executive Secretary of the Virology Panel, U.S.-Japan Cooperative Medical Science Program. Dr. Darrel D. Gwinn administers the Mycology and Mycobacteriology Programs, and also serves as Executive Secretary of the Leprosy Panel and of the Tuberculosis Panel, U.S.-Japan Cooperative Medical Science Program.

Approximate Level of Support

Bacteriology and Mycology

Activity	Number	Amount
Research Grants	194	$15,000,674
Research Program Projects	6	2,285,310
Career Awards	13	522,386
Training Grants	19	1,616,689
Fellowships	7	84,614
Research Contracts	13	479,406
Total	252	$19,989,079

Virology

Activity	Number	Amount
Research Grants	212	$17,767,663
Research Program Projects	2	682,541
Career Awards	19	648,981
Training Grants	12	1,095,478
Fellowships	21	311,591
Research Contracts	3	119,500
Total	269	$20,625,754

Branch

	Number	Amount
Total	521	$40,614,833

Program Summary

The program of this Branch focuses upon projects in both fundamental and applied biomedical research, with the ultimate and most significant objective being the translation of the knowledge gained into better and more practical methods for the diagnosis, prevention and therapy of bacterial, fungal and viral diseases. Sexually transmitted diseases and hospital associated infections remain as programs of Congressional and Institute special interest. The biology and biochemistry of viral infections of humans and animals continue as subjects of considerable research interest. The focus of this new research is on the mechanisms of viral replication and the regulation of expression of gene products, the relation of viral structural components to function, and the immune responses to infection. Pathogenetic mechanisms of acute viral diseases of man and mechanisms by which viruses can initiate and sustain persistent infections are areas of special concern and attention. Under the auspices of the U.S.-Japan Cooperative Medical Science Program certain disease areas have been singled out for special attention. These include research programs on tuberculosis and leprosy, as well as rabies, dengue and other arthropod transmitted diseases, infections that are still common in the world's developing countries.

Bacteriology

Sexually Transmitted Diseases (STD)

The sexually transmitted diseases constitute a major public health problem. No significant lessening of the overall problem is seen, although a slight leveling off of the gonorrhea rate has been noted. This disease continues as the major area of concern in STD, although it may well be superseded, according to many experts, by the increase in nongonococcal urethritis (NGU). Hard data are, however, lacking in these as in all the other STD problems. A significant increase in genital herpes is of concern to public health officials because of the potential for serious damage or death to infants born of mothers infected with herpes at the time of delivery. There is also increasing concern regarding other diseases, such as hepatitis, amebic dysentery and shigellosis, that are now being seen with increasing frequency, particularly among homosexual groups. The Institute's overall research program in support of the STD problem includes program projects (STD Centers), grants, contracts, and training.

Research Highlights

P01 AI 12192-05 K. K. Holmes (University of Washington): In this multifaceted project, Dr. Holmes' data indicate that specific auxotypes of gonococci (Arg Hyx Ura) are less often associated with pelvic inflammatory disease (PID) than with uncomplicated gonorrhea in women. Women who used oral contraceptives had a twofold greater risk of gonorrhea than did those using other contraceptives. This has important implications for the epidemiology of gonorrhea. Other data suggest that one episode of PID confers immunity to a second PID episode due to the same POMP type - the first example of immunity following a naturally occurring gonococcal infection. Dr. Falkow's studies on proteins isolated from Chlamydia trachomatis and C. psittaci revealed that five proteins from C. psittaci were immunologically related to C. trachomatis. Sera from infected humans contained 15 to 20 anti-chlamydial proteins. These studies may provide the future basis for specific serological tests for chlamydial infections. In other studies, Drs. Holmes and Alexander reported that erythromycin opthalmic ointment was significantly more effective than silver nitrate in preventing neonatal C. trachomatis ocular infections. Chlamydia also were reported to be a significant risk factor for post-partum endometritis; it was shown that cellular immunity to this organism was depressed during pregnancy.

N01 AI 92618 T. M. Buchanan (University of Washington): The development of a suitable vaccine for protection against gonorrhea is an important approach to the eventual control of this disease. New purification procedures for the isolation of principal outer membrane protein (POMP) of the gonococcal cell were developed; these resulted in POMP of greater antigenicity and in 40-fold higher yields. As is noted above, antibody to POMP provides immunity to recurrent salpingitis, indicating that POMP antigen should be included in a gonorrheal vaccine. Purified gonococcal pili as an immunizing antigen in humans induced antibodies that were highly opsonic; opsonic antibodies for gonococci are directed primarily against the pili antigens. The opsonic activity of the pili antibody is due to direct interaction in recognition of the Fc portion of the opsonic antibody by the Fc receptor on human phagoctyes. Both the pili antigen and the POMP antigen are under intensive investigation as candidate vaccine materials. The data obtained to date appear promising; in Phase 1 vaccine trials in volunteers good antibody titers, with minimal side reactions, have been observed.

P01 AI 15036-02 P. F. Sparling (University of North Carolina): This multidisciplinary project is directed toward basic studies of several important organisms causing STDs. Data from a study on lipopolysaccharide (LPS) antigen of the gonococcus showed that immunization with LPS could protect guinea pigs against challenge with the homologous gonococcal strain. Bactericidal response, correlated with subsequent immunoprotection, indicates that LPS may be a potentially important immunogen. Gonococcal POMP showed 100-fold or greater protection to homologous challenge in guinea pigs immunized with the corresponding POMP. Colonial morphology, especially the opacity-transparency phenotype, may be significant in determining the most important immunoprotective antigen. In other studies, data do not support the role of gonococcal L-phase variants in STD infections. The L-phase variants do not attach to cells in organ culture systems. Dr. Baseman's study of Treponema pallidum revealed that there are a limited number of binding sites for attachment of the organism. Virulent treponemes grown in vivo showed the presence of a surface coat of host serum proteins; some of these were loosely associated and readily removed by washing. Washed treponemes easily readsorbed numerous host proteins from serum. Other host proteins (IgG, ceruloplasmin, transferrin, etc.) were avidly associated and could not be removed from the treponemal outer envelope. These data suggest the presence of noncompetitive binding sites for serum proteins on the treponemal outer envelope. The association of numerous host proteins with the outer envelope of virulent treponemes could provide a mechanism whereby the pathogen might avoid immune surveillance or immune defenses.

AI 13069-05 A. L. Barron (University of Arkansas): The objective of this study is to develop an animal model for studying chlamydial genital infection, relating its pathogenesis to the human disease. Guinea pigs immunosuppressed with cyclophosphamide were infected intravaginally with the GPIC agent (a pig-adapted strain of C. psittaci). The resulting disease closely resembled infections in the human female, with labial edema and ulcerations, followed by endometritis and salpingitis. Intense chlamydial infection of the exocervix and endocervical mucosa were observed; fallopian tube involvement was found in 60% of the infected animals. Discontinuance of immunosuppression caused resolution of the infection. This model provides unequivocal evidence for ascending genital tract infection by the chlamydial agent, and also supports evidence for invasion of peritoneal tissue by chlamydiae.

AI 15642-02 G. F. Brooks (University of California, San Francisco): Dr. Brooks reports that human seminal plasma inhibits complement-mediated bactericidal and opsonic effects on the gonococcus and other gram negative microorganisms. This seminal plasma inhibitory factor is a low M.W. material that is a potent inhibitor of immune systems in the genital tract; hemolytic complement activity is reduced and C3 activation is blocked. Other data on the gonococcus revealed that the transparent colony phenotype is the invasive form of the organism. This phenotypic variant attached to genital tract cells or fallopian tubes much more than did the opaque colony phenotype. It was also reported that sera from women during different phases of their menstrual cycle showed differences in bactericidal activity.

Sexually Transmitted Diseases Study Group Meeting

The NIAID supported a Study Group on STD, held in January 1980, to review current research progress and make recommendations for needed further research in this area. A summary documenting the recommendations of the nine separate panels was presented for review to the National Advisory Allergy and Infectious Diseases Council. A brief summary will be published in the near future in the *Journal of Infectious Diseases*. The complete proceedings of this meeting will be submitted for publication by the Government Printing Office.

Hospital Associated Infections (HAI)

Hospital associated, or nosocomial, infections remain a public health problem of considerable magnitude. Although the data are incomplete, indications are that these infections result in significant mortality and prolonged hospitalization; excessive health care costs are the inevitable result. This problem will continue to increase as the immunocompromised segment of the population increases. The primary microorganisms involved are those of the gram negative group; many of them are merely opportunistic invaders. There is a disturbing trend to antibiotic resistance among these organisms, thereby causing continued problems in patient therapy.

Research Highlights

AI 10108-09 A. I. Braude (University of California, San Diego): Dr. Braude is continuing his studies on the J5 antiserum, prepared by immunization of human volunteers with the core glycolipid of an *E. coli* mutant strain. The immune serum has been used for prevention and treatment of gram negative bacteremia in nosocomial infections. Studies have been initiated in burn patients and neutropenic patients with leukemia or lymphoma for prophylactic protection against bacteremia. Results of the study in a randomized patient population showed that the J5 antiserum sharply reduced the incidence of fever from 44% in controls to 18% in treated patients; no bacteremia occurred in the treated patients. The lowered rate of fever may be from prevention of occult gram negative infections and endotoxemia.

In a continuing double-blind therapy trial with the J5 antiserum, analysis of current data showed a lowering of the death rate from 26% to 14%, and improvement in recovery from deep shock from 29% to 82%. The lung was the most common source of bacteremia, although the bowel and urinary tract are still important. The increasing prominence of *Klebsiella* species was also noted in these infections. Further data showed that J5 antiserum could protect against Hemophilus influenzae (the commonest cause of bacteremia and meningitis in infants). These results broaden the spectrum of gram negative infections amenable to therapy with the antibody to core glycolipid.

AI 12936-03 J. W. Alexander (University of Cincinnati): Nosocomial infections are a major unsolved problem in surgical practice. Data from Dr. Alexander's studies show that, in a burned dog model, administration of Corynebacterium parvum had a definite immunopotentiating effect. Significant improvement in overall survival and time to death was noted with use of C. parvum. No evidence of toxicity was observed in any of the test animals. Planned studies with C. parvum in human patients have now been initiated. Other studies on nutrition and immune status were completed; a marked benefit in burn patients was seen after high protein supplementation, with increased survival and improved immunologic functions. Research has revealed that defects in phagocytic functions are the predominant immunologic deficit associated with post-surgical infections; in many cases malnutrition seems to be the major contributing factor causing these deficits. Results of these studies may have a direct effect on current surgical practice.

AI 14533-02 R. W. Garibaldi (University of Utah): In this research project, data relating to post-operative pneumonias show that their incidence is influenced by a variety of host-related factors. Low levels of preoperative serum albumin is one such predisposing factor that can be readily modified in clinical practice. Data on the use of antibiotics in surgical practice in 21 Utah hospitals revealed that appropriate prophylaxis was used in only 14% of the 1,163 cases monitored. Most of the inappropriate usage centered on prolonged antibiotic administration. Antibiotics were also prescribed inappropriately in 27% of the surgical cases. The excess costs of inappropriate antibiotic use were estimated in this survey at $1.2 million per year. In a further study, a prevalence survey of Utah nursing homes revealed a high rate of nosocomial infections, primarily urinary tract and respiratory types. Twenty-three percent of these patients also were on inappropriate antibiotic therapy.

AI 15655-01 J. P. Burke (L.D.S. Hospital Foundation, Utah): Dr. Burke's studies have centered on reduction of morbidity and mortality attributable in some way to temporary use of indwelling bladder catheters. The use of closed sterile drainage techniques has not eliminated the problem; more than 20% of such patients continue to acquire bacteriuria. The major pathway for bacterial colonization is the periurethral mucous surface. The data indicate that extralumenal spread of bacteria within the periurethral space is the most important route by which bacteria gain entry into the catheterized bladder. The use of silicone rather than latex rubber catheters resulted in lower infection rates. Infections secondary to indwelling catheters account for more than 30% of all nosocomial infections, affecting more than 400,000 patients per year. Specific meatal prophylaxis was shown to be ineffective and potentially hazardous as a preventive method.

Streptococcal Diseases and Sequelae

Streptococcal diseases and their sequelae continue to constitute a troublesome health problem, particularly among the lower socioeconomic populations. The chief streptococcal disease problem in the U.S. is now group B streptococcal

(GBS) infections; these are a significant cause of neonatal and infant mortality and morbidity. Group A streptococcal infections and their serious sequelae, rheumatic fever and acute glomerular nephritis, although now somewhat less common in the U.S. and Europe, are nevertheless an important problem in many developing countries.

AI 08724-23 L. W. Wannamaker (University of Minnesota): This research group is studying the complex biology of the streptococci. Experiments on transduction of plasmids between group A and group G streptococcal strains suggest that an *in vivo* genetic exchange of resistance to clinically important antibiotics is possible. This may help explain observations about the increasing resistance to erythromycin among streptococci. Other results have shown that streptolysin O (SLO) is toxic to beating rat myocardial cells in tissue culture, causing myocardial cell fibrillation with cessation of beating and with bleb formation. The addition of cholesterol appeared to protect these cells from the SLO toxicity. After cholesterol treatment no cell deterioration or bleb formation was observed; one unit of SLO was neutralized by 25 mg of cholesterol. In studies with GBS cells, types Ia and Ib appear to adhere to pharyngeal or genital tract cells twice as well as the vaginal GBS strains types II and III. Purified DNAases of GBS strains showed no relationship between serologic types and production of a specific nuclease. The GBS nucleases are, however, immunologically different from the group A streptococcal nucleases.

AI 11161-08 C. Panos (Thomas Jefferson University, Philadelphia): Studies on effects of lipoteichoic acid (LTA) showed that LTA induced marked changes in mouse glomeruli. The capillary tufts shrank in size, and the glomeruli showed a greatly thickened basement membrane of non-uniform variability. This was confirmed using mouse kidney organ cultures treated with group A streptococcal LTA. Other studies showed that a neuraminidase-producing GBS strain could adhere to a variety of human cell monolayers; the LTA of this GBS strain was able to destroy human tissue culture monolayers. It was also shown that a streptococcal L-form of group A streptococcus no longer possessed a particular functioning amino acid transport system; its ability to transport glucose was also changed. These differences may help explain the differences in growth rates and cell yields between the streptococcal cell and its stabilized L-form.

AI 11822-06 V. A. Fischetti (Rockefeller University): Studies on the immunochemical structure of streptococcal M protein revealed that the M proteins share a number of physicochemical properties with mammalian tropomyosin protein. These findings of a striking primary structure and physicochemical similarity may aid in our understanding of the nature of a rheumatic fever attack. The poorly understood cross-reactive antibodies to smooth muscle tissues produced during rheumatic fever episodes may be the result of an immune response to this "mammalian-like" antigen on the streptococcal cell.

N01 AI 72538 D. L. Kasper (Peter Bent Brigham Hospital): Dr. Kasper and his collaborators are developing materials suitable for a candidate vaccine to prevent GBS infection in neonates. Type II GBS polysaccharide was found to have the same sugar structures as the previously defined type III and type Ia GBS antigens, but with somewhat different molar ratios. It was observed that antibody in human sera directed to the complete native polysaccharide antigen correlated highly with opsonic activity. The native type III GBS polysaccharide antigen (with the immunodeterminant sialic acid structure) appears to provide the best candidate antigens for immunization. The GBS type Ia candidate vaccine showed a somewhat lower level of seroconversion in individuals with normally low levels of pre-existing antibody, but was much higher for those with moderate levels of pre-existing antibody, whereas early data with the type II candidate vaccine indicate a high seroconversion rate. Both types Ia and II candidate vaccines have been safety tested in Phase I trials in volunteers.

AI 06487-15 D. W. Watson (University of Minnesota): The thrust of this research project has been an understanding of the streptococcal pyrogenic exotoxin (SPE). Dr. Watson described a toxin from staphylococcus with similar biologic properties to SPE; no antigenic relationship was, however, seen between these two toxins. In addition to fever production, both of the toxins produced enhanced susceptibility to lethal endotoxin shock, with damage to myocardium and liver. They also functioned as potent nonspecific lymphocyte mitogens; the target cell for the mitogenicity of both toxins is the T cell. While antipyretic drugs could protect rabbits from fever (SPE-induced), they could not protect from the enhancement of lethal endotoxin shock. The SPE toxin also apparently enhances hypersensitivity reactions, as manifested by skin rashes.

Other Disease Problems

There is an array of infectious disease problems caused by bacteria other than those singled out for special consideration that also merits attention. Many of these bacterial infections, once considered of little significance because of the widespread availability of antibiotics, remain as important diseases. Certain infections are now known to be more complex in their disease manifestations, while others have only recently been recognized as significant problems.

AI 08504-11 P. F. Bartell (New Jersey Medical School): The primary problem in cystic fibrosis (CF) is that of lung infection caused by *Pseudomonas aeruginosa* colonization. This microorganism produces, in the CF patient, a viscid slime composed of glycolipoprotein (GLP) that interferes with passage and absorption of antibiotics by the bacterial cell. After injection in mice, the purified GLP elicited the same toxic, leukopenic and lethal effects as did a lethal infection with viable bacteria. The polysaccharide moiety of the GLP, however, showed none of the toxic or lethal effects of the whole GLP. Coupling of the polysaccharide fragment to an albumen carrier plus Freund's adjuvant stimulated the production, in mice, of protective antibodies as shown by both active and passive immunization tests. Immunized mice were protected against lethal challenge; none of the controls survived. The purified polysaccharide fragment of GLP may offer some hope for an eventual candidate vaccine material.

AI 15835-01 G. Pier (Peter Bent Brigham Hospital): A clearer understanding of the many discrepancies regarding toxicity, specificity and host responses of pseudomonas toxins is needed. Dr. Pier reports that the organisms, grown in large volume cultures, yielded significant amounts of carbohydrate antigens. Purified antigens from seven different immunotypes showed the presence of galactose, mannose, glucose, rhamnose, and other sugars. The monosaccharides were not present in the lipopolysaccharide (LPS) from these organisms. Immunization of mice with preparations from two pseudomonas immunotypes was effective in protecting against challenge with live organisms. The "O" side chains of an LPS preparation were poorly immunogenic in eliciting protection against challenge with virulent organisms. The immunogens eliciting protection were effective against both homologous and cross-immunotype challenge. These studies should provide data that may ultimately lead to safe and effective candidate vaccines for pseudomonas infections.

AI 15903-01 H. N. Beaty (University of Vermont): The thrust of this research is on the pathogenesis of Legionnaires' disease and host defenses, in a guinea pig model of pulmonary infection. A serogroup I strain of *Legionella pneumophila* isolated from a patient was grown to high titer (10^9/ml) in a biphasic medium. The organisms, after inoculation at different dilutions into the animals, produced fatal pulmonary infection with extensive, diffuse bilateral bronchopneumonia. No extrapulmonary infection was seen. A microagglutination technique was developed for measuring serum antibody responses; titers were comparable to those measured by immunofluorescent antibody techniques. These early studies indicate that *L. pneumophila* grown in broth culture is virulent for guinea pigs by the intrapulmonary (IP) route. Studies on pathogenesis and host responses to the infection are now well underway.

AI 07826-11 F. A. Kapral (Ohio State University): This project's objective is to obtain a better understanding of host-parasite interactions in staphylococcal infections. A bactericidal substance, found in staphylococcal abscesses in mice, was identified as a fatty acid (FA) comprising 1% of the FA pool in the abscess; it is not present in normal tissue. Staphylococcal strains differ markedly in their sensitivity to the bactericidal FA and their ability to survive within the abscess. The bactericidal FA is generated from a lipid precursor developing within the abscess but is not present in normal or adipose tissue. The bactericidal FA was also found in mouse lung after staphylococcal infection. In the kidney, which may be a privileged site of staphylococcal growth, an inhibitor of the bactericidal FA is found. The inhibitor, also an FA, is distinct from the bactericidal FA. Neither the precursor nor the bactericidal FA was detectable before 12 hours. The bactericidal FA increased steadily for seven days before leveling off; by the 14th day the concentration had dropped to a low level. The bactericidal FA is present in the neutral lipid fraction of infected mouse kidneys whereas the inhibitory FA resides in the phospholipid fraction. In current studies the principal investigator is attempting to fractionate and identify the bactericidal FA; it does not appear to be a common FA and the precursor may be a neutral lipid. The bactericidal FA may function as an immune mechanism and it may also play a role in other infections.

Leprosy

Leprosy cannot be considered a major health problem in the United States since only approximately 150 new cases are reported yearly, although it is estimated that there are as many as 3,000 active cases of leprosy in the United States. The duration of the disease, its prolonged costs, and its social and human consequences, however, are such that even a few cases have far-reaching impact. Leprosy is a significant health problem in the developing countries of the world; the majority of the estimated 12 to 15 million cases are found in Asia, Africa, and South America. Research in leprosy is supported by NIAID under the U.S.-Japan Cooperative Medical Science Program.

Research Highlights

AI 08214-13 J. H. Peters (Stanford Research Institute): Using high pressure liquid chromatographic procedures, Dr. Peters has continued his studies on the metabolic disposition of antileprosy drugs in animals and man. He had previously determined the metabolic disposition of dapsone, clofazimine and rifampin; now he has determined the metabolic disposition of ethionamide and prothionamide in animal models, using rats and armadillos. The thioamides have become firmly established as bactericidal drugs against M. leprae in animals and man. He has also found that volunteers receiving oral dapsone exhibited consistent, predictable plasma/saliva ratios, demonstrating that patients could be monitored for dapsone intake employing saliva obtained noninvasively.

AI 07801-13 J. L. Krahenbuhl (U.S. Public Health Service Hospital, San Francisco): The objective of Dr. Krahenbuhl's research is to investigate the immunology and immunotherapy of experimental leprosy. In studies to determine the efficacy of adjuvant immunotherapy, he has investigated the effects of the synthetic adjuvant muramyl dipeptide (MDP) and its analogs. When administered in oil, MDP appears to be the smallest chemical unit that can replace the effects of the mycobacterial cell wall in Freund's complete adjuvant. When administered in an aqueous solution, toxic side effects are few and minimal, and MDP can markedly enhance both antibody formation and cell-mediated immunity to unrelated antigens. However, in some initial studies, neither pretreatment with MDP nor treatment administered at varying times after infection prevented growth of M. leprae or M. marinum in the mouse foot pad. Dr. Krahenbuhl is initiating more definitive studies of the efficacy of this form of immunotherapy in leprosy.

N01 AI 92624 T. M. Buchanan (U.S. Public Health Service Hospital, Seattle): Dr. Buchanan is concerned with the isolation, purification and characterization of protein antigens from leprosy bacilli purified from armadillo tissue, with the specific aim of identifying M. leprae antigens not found in other mycobacteria. His purification procedure results in a reduction from some 14 major protein bands to two major protein bands in the purified preparation, as assessed by polyacrylamide gel electrophoresis. The subunit molecular weights of the two proteins from M. leprae that carry M. leprae-specific activity are approximately 35,000 and 20,000 daltons. It appears that antibodies of the patients are directed at each of these two proteins. When tested against serum from leprosy patients, only M. lepraemurium and one strain of M. bovis showed some reactivity. The antigenic extracts of these two mycobacteria were further purified using the same methodology used to purify the M. leprae-specific protein antigens. These extracts showed the presence of a 35,000 dalton protein but no 20,000 dalton protein. This suggests that the antigenic specificity for M. leprae is resident on the 20,000 dalton protein or is present along with other antigenic determinants that are shared with M. lepraemurium and M. bovis on the 35,000 dalton protein. Dr. Buchanan will now concentrate on obtaining the 20,000 dalton protein from M. leprae and determine if it contains the antigenicity that is specific for M. leprae.

N01 AI 92625 P. J. Brennan (National Jewish Hospital, Denver): Dr. Brennan is concerned with the isolation, purification and characterization of lipid antigens from leprosy bacilli purified from armadillo tissue. Lipid extracts obtained from purified M. leprae were sonicated and tested for serological activity against antisera from normal and M. leprae-infected armadillos. These lipid extracts gave distinct lines of precipitation with the serum from infected armadillo and did not react with the serum from the normal animals. Partial purification of the lipid extracts was achieved. The partially purified lipid from M. leprae yielded two very definite lines of precipitation with sera from lepromatous patients and with that from the infected armadillo. There was no reaction to the sera from patients with tuberculosis or M. avium infection, or to the sera from the normal armadillo. These preliminary experiments give reason to believe that M. leprae contains species-specific lipid antigens, a phenomenon which is very common in most atypical mycobacteria. Experiments are now underway to further examine the specificity of this lipid antigen and to characterize it chemically.

Tuberculosis

Tuberculosis cases and deaths in the United States have declined steadily during this century. The incidence of tuberculosis cases varies broadly among different segments of the population and in different localities. In Washington, D. C., for example, the incidence has climbed to more than triple the national average. Tuberculosis in the developing nations of the world remains one of the major causes of morbidity and mortality in all age groups. Research on tuberculosis is supported by NIAID under the U.S.-Japan Cooperative Medical Science Program.

Research Highlights

AI 15102-02 T. M. Daniel (Case Western Reserve University): Mycobacterium kansasii is a well-recognized cause of chronic granulomatous disease. The clinical, bacteriologic, radiographic and pathologic features of disease due to this organism have been identified in recent years. There are few data, however, on the immunology and epidemiology of M. kansasii infections. Dr. Daniel has identified a highly anodal protein antigen, with a molecular weight of approximately 10,000 daltons, in culture filtrates of M. kansasii. This antigen was not found in culture filtrates of nine other mycobacterial species. Using immunoabsorbent affinity chromatography, this antigen has been purified and used to skin test guinea pigs sensitized with M. kansasii and M. tuberculosis. In these animals it was found to elicit delayed hypersensitivity reactions and to be at least partially species-specific for M. kansasii.

AI 15049-01 P. R. Gangadharam (National Jewish Hospital, Denver): Peritoneal and alveolar macrophages were compared with respect to the pathogenesis of M. intracellulare. Significant differences between the two types of macrophages were noted when they were assessed according to phagocytic index, frequency distribution of ingested bacilli and intracellular growth rate. The alveolar macrophages are significantly slower (up to 50%) than the peritoneal macrophages in initiating ingestion of the bacilli during the first 12 hours of incubation. Both the frequency distribution and the rates of intracellular multiplication also differ significantly between the two cell types.

Mycology

Fungi such as Histoplasma capsulatum and Coccidioides immitis are primary pathogens in normal persons, while fungi that cause systemic, lymphatic, and subcutaneous mycoses are normally called "opportunistic fungi." The term "opportunistic fungi" is used to emphasize that a normally saprophytic fungus can suddenly become parasitic and pathogenic when it is introduced by inhalation or trauma into patients with impaired host defense mechanisms. Candida albicans and Cryptococcus neoformans are examples of the important "opportunistic fungi." Amphotericin B is the drug most commonly used in the treatment of these fungal infections. Treatment requires prolonged administration, and side effects are common after administration of this toxic drug. The Institute is presently funding research in which the primary fungal pathogens and the "opportunistic fungi" are being investigated.

Research Highlights

AI 15996-01 J. E. Cutler (Montana State University): Dr. Cutler has found that Candida albicans produces a morphogenic autoregulatory substance (MARS) when grown in certain tissue culture media that keeps the organism in the yeast phase. He has also isolated an ultraviolet-induced mutant of C. albicans that develops mycelia but does not convert to the yeast phase. It is known that both yeasts and hyphal elements are present in candidal lesions and this has led to a great deal of speculation regarding the role of either phase in the pathogenesis of the disease. This MARS factor along with C. albicans mutants may become a major tool in understanding the mechanism and control of yeast-to-mycelia conversion.

AI 13167-05 B. L. Beaman (University of California): Dr. Beaman has been investigating mechanisms controlling nocardial pathogenesis. His studies on the role of L-phase variants of Nocardia caviae in disease have shown that these microbial variants are readily induced in the intact murine lung where they produce an acute pneumonia that results in death. Also, he has shown that L-phase variants can persist in the host for at least two years and eventually lead to progressive disease from which only the L-phase variant and not the parental bacteria could be isolated. When L-forms of N. caviae were induced in vitro and then injected into mice, it was shown that after six months to one year they produced progressive disease. Thus, L-forms of this organism may play an important role in the disease process as they appear to be pathogenic for mice.

AI 15682-02 J. M. Jones (University of Wisconsin Medical School): Dr. Jones has developed a radioimmunoassay to measure levels of antibody against cell wall mannan of Candida albicans. Two patients with acute leukemia were diagnosed as also having granulomatous hepatitis. C. albicans could not be isolated from liver biopsies taken from these patients, but yeast and filamentous fungal forms could be identified histochemically within the granulomas found in the biopsy specimens. Quantitation of anti-C. albicans antibody levels confirmed that deep-seated C. albicans infection had occurred in both patients. It had not been appreciated that C. albicans could produce granulomatous hepatitis until these studies were done.

AI 05022-16 G. S. Bulmer (University of Oklahoma): During studies on the pathogenesis of cryptococcosis, mice were immunized with a viable, avirulent strain of Cryptococcus neoformans. Lymphocyte blastogenic assays showed a tenfold increase in reactivity of sensitized spleen cells, and histopathologic examination revealed marked splenic hyperplasia. Thirty-two days after intravenous inoculation with a virulent strain of C. neoformans, none of the control animals survived whereas 60% of the immunized mice were alive with no clinical evidence of disease. This animal model shows that protective immunity can be established and can provide a better model for the study of important aspects of immunity in fungal disease.

Virology

Clinical Virology

Herpes infections of man continue to dominate this program of special Institute interest. Research with clinical relevance to the pathogenesis of acute viral infections is encouraged to bring the clinician closer to new information and techniques generated by academically oriented virologists. Increasing concern and interest are being devoted to genital herpes (herpes simplex type II) infections because of the alarming increase in incidence. Oral facial herpes (herpes simplex type I), infectious mononucleosis, cytomegalovirus infections, and varicella-zoster are other herpesvirus infections commanding more and more attention and interest by both virologists and clinicians.

Research Highlights

AI 14741-01 D. Horstmann (Yale University): Previous studies in this laboratory have supported the concept that Epstein-Barr virus (EBV), the putative agent of infectious mononucleosis, is enveloped by a membrane derived from infected cells and is released by some cells into the mouth and throat. In current investigations the virus was found to be shed in cell-free form into the saliva only by cells of the parotid gland. Surprisingly, the virus was not expressed in tissues of the tonsils, which are rich in lymphocytes. The virus can usually be activated from its latent form by in vitro cultivation of circulating blood lymphocytes but these tissues do not shed cell-free virus. From a practical standpoint, salivary gland cells, maintained in tissue culture, might be induced to release latent EBV or serve as a source for propagation of the viru in vitro.

AI 15310-01 D. Thorley-Lawson (Sidney Farber Cancer Institute): Dr. Thorley-Lawson's studies involve the interaction of EBV and lymphocytes; lymphocyte proliferation is a clinically recognizable sign in infectious mononucleosis. He has demonstrated that thymus derived lymphocytes (T cells) suppress the outgrowth of the infected B cells within 24 hours post-infection, before the expression of the virus through stimulation of DNA synthesis and appearance of viral antigens. The T cells become much less effective at suppressing the proliferation of bone marrow-derived lymphocytes (B cells) once they are transformed by the virus. T cells do not affect the virus itself nor do they interfere with viral absorption and penetration. The conclusion is that the T cells are responding to viral antigens left on the surface of newly infected cells in such a way as to prevent infected cells from transforming and proliferating.

AI 12814-04 A. E. Gershon (New York University Medical Center): This group of clinical investigators has now applied successfully three different assays for cell-mediated immunity (CMI) to varicella-zoster (V-Z) virus. CMI was depressed in immunocompromised persons in comparison to normal persons years after a V-Z infection. These results suggest the importance of CMI in active and latent V-Z infections. Older persons, who are at 10 times the risk of the young to develop zoster, had higher antibody titers to V-Z virus than did younger patients and there was no difference between antibody titers in normal and immunocompromised persons. Therefore, decrease in antibody titers is not a predisposing condition for reactivation of latent V-Z infections.

AI 15491-01 J. Oakes (University of South Alabama): Using mice as a model for ocular herpes infections of man, Dr. Oakes has found that specific antibody could inhibit the spread of virus following primary ocular Herpes Simplex Virus (HSV) infection in an immunocompetent individual; a radiation-sensitive component, however, was required for antibody-dependent immunity to be effective in protecting the host from a fulminating infection. These results emphasize the complexity of interacting components of the immune system in resistance to HSV infections.

AI 14981-02 B. T. Rouse (University of Tennessee, Knoxville): Dr. Rouse has developed an animal model for studying reactivation of latent HSV. Mice were inoculated with sublethal doses of HSV intradermally into the external ear. After a period of six weeks recrudescent lesions could be produced and virus isolated from 75% of the animals by stripping the ear repeatedly with plastic tape. This model may allow the investigators to define the host factors that control latency and reactivation.

Persistent Viral Infections and Chronic Diseases

Establishment of persistent viral infections may be achieved by various mechanisms controlled either by the host or directed by the virus. The elucidation of these mechanisms and the immunopathogenic responses leading to clinical disease are the goals of this program. Recrudescent or chronic diseases that are associated with persistent infections include herpes simplex infections, cytomegalovirus infections, herpes zoster, subacute sclerosing panencephalitis, hepatitis B, arenavirus infections and several spongiform encephalopathies.

AI 11217-07 D. L. Walker (University of Wisconsin): Dr. Walker is studying JC virus, a human papovavirus associated with chronic, degenerative central nervous system disease. Cell-mediated immunity to JC virus (JCV) was assessed by production of leukocyte-migration-inhibitory factor (LIF) in response to JCV antigen. In the PML patients tested, LIF production with JCV antigen was absent despite the presence of antibody to JCV in serum. This contrasted with positive LIF production in seropositive normal individuals and patients with other diseases. These results provide the first *in vitro* evidence of depressed cell-mediated immune response to JCV in patients with PML, and support the hypothesis that PML is accompanied by a selective and marked deficiency in cellular response to this virus, in association with a general depression of cell-mediated immunity of variable severity.

AI 14627-12 J. J. Holland (University of California, San Diego): Although a role for defective interfering (DI) particles in persistent infections by rhabdoviruses and other negative positive strand viruses has been clearly demonstrated in recent years, unequivocal demonstration of DI particles in persistent infections by measles, mumps and other paramyxoviruses has been difficult to accomplish. Dr. Holland has now shown that for the mouse paramyxovirus, Sendai virus persistence in tissue culture cells requires the presence of DI particles for rapid establishment of persistent infection. Analysis of the viral ribonucleoprotein particles within persistently infected cells showed that there is always a vast excess of DI particle RNA genomes over standard virus genomes in the cytoplasm. Only tiny amounts of mature DI particles and of standard virus were shed from the persistently-infected cells, and these were biologically inactive unless they were activated by trypsin treatment.

AI 12203-05 F. Rapp (Pennsylvania State University, Hershey): A persistent measles virus infection was established in a human amnion cell line. While certain characteristics of the system were similar to those expressed by previously reported persistent measles virus infections, distinct differences were also noted. Persistently infected cells passed through two unstable phases ("acute" and "chronic") before stabilizing in a third phase ("latent"). In the first two phases, the infected cells went through degenerative cytopathic and cytocidal crises during which relatively high levels of infectious, cytopathic particles were continuously shed. In the third phase, the cultures expressed no viral cytopathic effects, particle shedding was greatly reduced, and infectious particles that were shed demonstrated significantly attenuated cytopathology. The production of significant amounts of defective-interfering particles was not observed in any phase of the persistent infection. Ultrastructural studies performed on the three different phases of persistent infection showed a shift from "fuzzy" nucleocapsids in the cytoplasm (acute and chronic phases) to "smooth" nucleocapsids (in the latent phase). These studies also showed that two of the major structural virus polypeptides produced by the latent virus isolates were distinct from those produced by the original (or acute) input virus. Results of these studies indicate that virus variance with attenuated biological activities and modified structural proteins arise in certain persistent measles virus infections; they further indicate that altered virus functions are involved in the establishment and maintenance of in vitro persistent infections. Dr. Rapp has obtained evidence that non-viral variables may play a role in blocking certain aspects of normal virus replication, thereby leading to persistence. These observations are significant because they indicate that the host cell as well as the virus may be involved in viral persistence.

Biology of Viruses

From the accumulated body of information on viruses it has become apparent that effective methods for treating or preventing viral diseases in man may be impossible without a thorough understanding of virus structure and of how viruses are able to infect, replicate and elicit disease. Through such studies, technologies are being developed that allow viruses to be used as probes for understanding normal metabolic processes of mammalian and bacterial cells.

AI 12717-05 E. S. Huang (University of North Carolina): Through very sensitive molecular probes Dr. Huang has detected human cytomegalovirus (CMV) DNA in normal cervixes and in cervical cancers from various geographic regions. CMV DNA could be detected in cervical cancers from Taiwan, Africa, Finland and the United States. CMV DNA is found frequently (about 50%) in normal cervixes in Taiwan and Africa, but less so in women from Finland. Only among the Taiwanese was frequency of the existence of CMV viral genome in cervical cancer significantly higher than in normal controls. DNA of genital herpes simplex virus was detected only in two cervical specimens; one from cervical cancer and one from a normal cervix of an African. The results of these studies suggest that CMV is much more prevalent in cervical tissue among many ethnic and geographic populations than is genital herpesvirus. These findings detract from the current hypothesis that genital herpesvirus is associated with the induction of cervical cancer. The present evidence is not convincing that either herpes simplex virus or CMV induce human cervical carcinomas.

F32 AI 05817-01 D. M. Coen (Sidney Farber Cancer Institute): When herpes simplex virus (HSV) infects cells it normally induces the cell to produce a virus-specific thymidine kinase (TK), an enzyme necessary for DNA replication. Using a cell line deficient in this enzyme in the presence of acycloguanosine - Acyclovir (ACG) - an anti-herpes drug of considerable clinical promise, Dr. Coen has been able to isolate HSV mutants that express decreased levels of TK. This drug kills the virus while doing no apparent damage to host cells; available evidence indicates HSV TK is essential for the drug's antiviral effect. All of the mutants selected behave in genetic mapping experiments as alleles of a single, recessive locus. Dr. Coen has also elucidated some new information on the mechanism of action of ACG by the demonstration that two mutants that are resistant to ACG induce DNA polymerase activities that are controlled by a gene other than the one regulating TK activity. These findings also raise the issue of the development of viral resistance to ACG in clinical use.

AI 10752-08 D. S. Ray (University of California, Los Angeles): Dr. Ray has been determining the biochemical mechanisms involved in the regulation of replication of one of the smallest known DNA viruses, bacteriophage M13. Current research has established the location of several genes associated with DNA replication, and provided a clearer and more detailed understanding of the structure and regulation of this small bacterial virus. Its unusual physical structure has allowed investigators to insert additional foreign DNA into this small viral chromosome. Chimeric M13 phages carrying replication origins from other phages and from the bacterial chromosome are proving to be valuable systems for the analysis of the role of specific DNA sequences in DNA replication. Because viral and cellular DNA replication are regulated primarily at the level of DNA initiation, these systems provide a new and powerful means of understanding one of the most important regulatory controls in the growth of viruses and tissues.

H 900-67X K. Johnson (Center for Disease Control, Atlanta): A combined medical and scientific team from CDC is determining various demographic parameters of the mouse *Mastomys natalensis*, the reservoir host of Lassa fever virus in Sierra Leone. The objective of this research is to elucidate sufficient information about the natural history of this virus and its natural host to be able to control effectively its transmission to man. Current studies have revealed that of the two karyotypes of *M. natalensis* found in Sierra Leone both the 2N=32 and the 2N=38 types harbor the virus. Both karyotypes are found among the village houses in the Northern Province. The 2N=32 karyotype, however, predominates in the Eastern Province, where a much higher rate of infection is found in villages in the diamond mining areas. Studies have thus far revealed that only the *Mastomys* and man are infected with Lassa virus. There being no strong seasonal pattern noticeable in *Mastomys* breeding, it is tempting to relate this pattern with the observed continous endemic transmission of human Lassa fevers, but further data are needed to confirm this preliminary correlation.

Virology Research under the Auspices of the U.S.-Japan Cooperative Medical Science Program

Goals for this program include the encouragement and support of research that will advance our knowledge of and lead to eventual control of rabies, dengue and other arthropod-borne diseases and viral gastroenteritis. The program is monitored by the Panel on Viral Diseases, an advisory group to the U.S.-Japan CMSP.

Research Highlights

AI-09706-09 H. Koprowski (The Wistar Institute): The new human diploid cell culture rabies vaccine that was developed by the Wistar Institute is now licensed for use in the U.S., replacing the older, less antigenic duck embryo vaccine. The new vaccine reduces the number of inoculations required for post-exposure treatment of potential rabies infections from 23 to 5. Wistar investigators have recently been studying the antigenic composition of the rabies virus by use of monoclonal antibodies. Twenty-five different cloned monoclonal antibodies directed against the glycoprotein of rabies virus were classified into 14 antigenic groups. It was found that some monoclonal antibodies failed to neutralize different virus strains even though they produced a positive reaction in other serologic tests. No satisfactory explanation has been given to this observation at the present time, but it was concluded from these studies that there is antigenic diversity among several strains. These results strongly suggest that the failures in the vaccine treatment of exposed persons may be due to existence of antigenic differences among the strains of rabies virus.

AI 10735-06 E. Bohl (Ohio Agriculture Research and Development Center): In cooperation with and under the direction of NIAID intramural staff this group successfully passaged serially human rotavirus type 2 in gnotobiotic pigs so that the virus might adapt better for growth in cell cultures. This was the first time that human rotovirus, one of the putative agents responsible for severe diarrhea in infants, has been grown in cell culture. This major breakthrough in technology is considered a prerequisite for possible future vaccine development.

CLINICAL STUDIES BRANCH

The Clinical Studies Branch (CSB) serves as the Institute's focus for the development and processing of Investigational New Drug Applications, and maintains liaison with the NIH Human Research Review Panel and the NIAID-Clinical Research Subpanel (CRS). It provides and monitors a closed clinical facility for Institute volunteer studies, and conducts, promotes and supports the development and evaluation of procedures for the diagnosis, prevention and treatment of infectious diseases, particularly those procedures leading to improved patient care.

Approximate Level of Support

Activity	Number	Amount
Research Grants	2	$132,388
Research Contracts*	6	$1,638,898
Training Grants	0	0
Fellowships	0	0
Total	8	$1,771,286

* One contract has been forward-funded with FY 1978 funds not shown here.

Program Areas

Closed Volunteer Facility

The CSB currently supports one research contract with the University of Maryland. This contract maintains and supports the Center for Vaccine Development (CVD) where volunteers are studied before and after they are challenged with potentially transmissible infectious agents and candidate vaccines. During the past year, the contractor performed twenty-five in-patient and four out-patient studies involving 252 volunteers. These studies were carried out under nine active protocols approved by the Institutional Review Boards of both the University of Maryland and NIAID. Included were *Escherichia coli* diarrhea (12 studies, 3 protocols); cholera (9 studies, 3 protocols); viral diarrhea (1 study, 1 protocol); and influenza (7 studies, 2 protocols). Highlights of these studies in volunteers are summarized below.

In the course of combined *E. coli* challenge and attenuated influenza vaccine studies carried out in tandem on the Isolation Ward of the CVD, it was shown that person-to-person contact transmission of enterotoxigenic *E. coli* does not occur. Lactinex, sold widely throughout the world as an unproven prophylactic against diarrhea, was not effective in preventing enterotoxigenic *E. coli* diarrhea in volunteers. Studies were continued with purified type 1 somatic pilus vaccine from *E. coli* H10407. The vaccine has continued to be non-reactogenic, and an optimum dosage regimen has been developed with which to determine vaccine efficacy against homologous and heterologous *E. coli* challenge.

Volunteers challenged 34 to 37 months earlier with a classical Ogawa serotype of Vibrio cholerae were fully protected against re-challenge by the same organism. This study provides direct evidence that immunity conferred by "natural" infection with classical V. cholerae lasts at least three years. A naturally occurring, non-toxigenic El Tor cholera strain isolated from waste water in Brazil did not produce diarrhea in volunteers, although 40% of subjects were infected with the organism. None of these infected volunteers were protected against challenge 4 weeks later with virulent El Tor, demonstrating that one dose of "attenuated" Brazil strain conferred no immunity. Two laboratory derived mutant strains of V. cholerae, Texas Star-SR (toxin A^- $B+$) and tox-101T1-N4 (toxin $A+$ B^-) were evaluated in volunteers for reactogenicity, colonization and immune response. The tox-101T1-N4 strain did not infect volunteers. The Texas Star strain did infect some volunteers and mild illness was noted in 18% of volunteers regardless of dose. Six of eight volunteers immunized with Texas Star were protected from reinfection after challenge with a partially attenuated parent strain of Texas Star. Possible protection by Texas Star against a diarrheagenic challenge will be tested in the near future.

In collaboration with the NIAID Laboratory of Infectious Diseases, studies were carried out with the Marin County agent, a 27nm particle suspected of producing a gastroenteritis outbreak in California. One of two volunteers developed diarrheal illness; laboratory results are pending.

Volunteers were inoculated intranasally with A/Alaska/6/77 CR31 clone 3 and clone 10 attenuated influenza vaccines. These cold adapted, attenuated mutants were generally non-reactogenic at challenge doses of $10^{7.5}TCID_{50}$. An altered temperature sensitive virus, A/Alaska/77 Ts-1A2, recovered from a child volunteer, was reactogenic in some adult volunteers at doses of $10^{4.0}$ and $10^{5.0}$ $TCID_{50}$, suggesting that Ts-1A2 strains are less suitable vaccine candidates than cold adapted strains. A dose-response challenge study was carried out with A/California/10/78 (H_1N_1) to determine virulence. Three of five volunteers developed influenza illness during this study.

Collaborative Clinical Trials Of Antibiotic Therapy

The major goal of this program is to sponsor controlled clinical trials designed to improve medical care of patients with bacterial and mycotic infections. Approved antibiotics, or promising new drugs that lack the economic potential to justify testing by the pharmaceutical industry, are evaluated for efficacy and safety in patients with selected infections.

A contract with the University of Alabama to study therapy of cryptococcal meningitis has provided useful clinical and laboratory data during its second year of operation. The first part of this project includes a controlled clinical trial by 13 collaborating medical centers to determine the shortest treatment regimen utilizing Amphotericin B (AMB) and 5-fluorocytosine (5-FC) in combination. Four and six weeks treatment regimens are being compared for efficacy and toxicity. Patient enrollment continues to fall short of that initially anticipated. This unexpected lag in achieving the desired sample size is due to the unexpectedly low occurrence of the disease and a necessary modification of the protocol to exclude renal transplant patients. In November, 1979, after noting the excessively high relapse rate for renal transplant patients on the four week regimen, the Study Monitor Group recommended that renal transplant patients only be enrolled into the six week regimen. This type of high-risk patient is extremely difficult to manage clinically and so comprehensive data on the outcome of renal transplant patients with cryptococcal meningitis will be a useful contribution to the field. Additional cases will be sought by extending the duration of the trial and adding several institutions to the Collaborative Group.

A second part of this project includes a protocol to conduct phase II studies with ketoconazole (R41,400), a new oral imidazole derivative with broad-spectrum antimycotic activity developed by Janssen Pharmaceutica of Belgium. There has been excellent progress in evaluating this new agent in the following group of systemic mycotic diseases: chronic cavitary histoplasmosis, disseminated or localized blastomycosis, disseminated coccidioidomycosis without meningitis, disseminated sporotrichosis, and non-meningeal cryptococcosis. A total of 44 patients have been enrolled, and there has been no serious toxicity associated with the administration of ketoconazole in the dose range of 200 - 600 mg. Final data on efficacy are not yet available.

Through a subcontract with the University of Texas, San Antonio, investigators are conducting combination drug studies in a cryptococcal meningitis mouse model. The combination of ketoconazole and 5-fluorocytosine has shown promise in preliminary studies. Investigators at Duke University have developed a cryptococcal meningitis rabbit model to assess the efficacy of combination drug therapy. Based on preliminary data, these investigators suggest that the combination of AMB plus ketoconazole is more efficacious than AMB alone or than the combination of AMB plus 5-fluorocytosine.

During FY '80 a contract was initiated with the University of Illinois to perform a placebo-controlled, double-blind evaluation of the efficacy of penicillin in the prevention of early onset Group B Streptococcal (GBS) sepsis in the newborn. Other objectives of this project are to determine if penicillin prophylaxis influences the rate of late onset GBS disease, increases or decreases the likelihood of later penicillin hypersensitivity, or causes the emergence of penicillin resistant GBS or other penicillin resistant organisms. Progress for the first year was slower than projected because of difficulty in recruiting volunteer mothers and the necessity to appoint a new Project Director for the trial.

Staff of the CSB were instrumental in planning the conference on "Treatment of Infective Endocarditis" held at the American Heart Association National Center, Dallas, Texas on April 28 - 29, 1980. The conference was co-sponsored by the NHLBI. A major purpose of the meeting was to provide an *ad hoc* committee the opportunity to arrive at a consensus on the optimal therapy of *Streptococcal viridans* endocarditis. Infectious disease specialists also focused on the treatment of infective endocarditis due to enterococci, staphylococci, fungi, as well as prosthetic valve endocarditis. A draft consensus document with specific recommendations was developed by the *ad hoc* committee. Assuming the councils of the American Heart Association (AHA) endorse these recommendations, these guidelines will become available to American practitioners through the extensive network of the AHA.

To stimulate the field of clinical trials, staff prepared a program announcement entitled, "Controlled Clinical Trials for Prophylaxis or Improved Antimicrobial Therapy of Selected Bacterial and Mycotic Infections." This announcement was reviewed and enthusiastically approved by the NIAID Advisory Council at the May 22, 1980 meeting. It is anticipated that it will be released in the NIH Guide for Grants and Contracts during the last quarter of FY '80.

Staff of the CSB accepted the responsibility for planning and organizing a "NIH Consensus Development Conference on Outmoded Antibiotics." The major thrust of this proposed conference will focus on making the scientific community and public aware of the problem of outmoded antibiotics, their impact on medical care and cost, and the processes, or lack of them for regulating their use by practitioners. This one or two day conference is tentatively scheduled for April, 1981.

Rapid Viral Diagnosis

The ultimate goal of this program is to provide the physician with reliable tests so that a specific viral diagnosis can be made in time to help in the management of the patient. This requires that the methods be practical, precise, reproducible, and inexpensive and that the viral antigen or antibody being measured appear in an acute clinical specimen. Currently there are two grantees and three contractors working to devise such tests. One grantee at Yale is studying methods to diagnose La Crosse (LAC) arboviral encephalitis, including an indirect immunofluorescent antibody spot slide test, an enzyme-linked immunosorbant assay (ELISA) and an enzyme-linked fluorescent assay (ELFA) for LAC virus. Another grantee at Baylor projects that the ELISA will detect less than 30 $TCID_{50}$ of influenza virus in his model system.

Three contractors are studying various parameters of the ELISA and its modification. Dr. Herrmann at Harvard evaluated different methods of covalently immobilizing capture antibody to solid surfaces for influenza virus detection. An advantage of covalently attached antibody is that the beads he uses can be washed and re-used. Dr. Richman, at the University of California, San Diego, has been applying his immunofiltration manifold and radioiodinated staphylococcal protein A (SPA/RIA) technique to the detection of herpes and myxovirus antigens and antibodies. He can detect and identify approximately ten infected culture cells that are either intact or disrupted, or 10^4 gradient-purified plaque forming units. His varicella antibody test differentiated among children susceptible to chicken pox, those who had received varicella immune globulin and those who had recently recovered from chicken pox.

Dr. Yolken at Johns Hopkins University has pursued several lines of investigation during the past year, including improvement of existing enzyme-linked immune assays for virus and antibody detection and the initiation of an epidemiological study of viral infections at the Johns Hopkins Hospital Infant Special Care Unit. The work of Dr. Yolken and his collaborators during the past year have resulted in 10 papers published or in press.

On May 8 and 9, 1980 the CSB and the Pan American Group for Rapid Viral Diagnosis co-sponsored their second workshop on New and Useful Techniques in Rapid Viral Diagnosis. More than 200 persons attended the meeting held at the NIH, indicating the interest in this rapidly developing field. A summary of the workshop will be published in the Journal of Infectious Diseases.

Dr. Robert Edelman presented the status of the NIAID's program on rapid viral diagnosis at a "Workshop on Rapid Identification of Bacterial Warfare Agents" held at USAMRIID, Fort Detrick on May 6, 1980.

Nutrition, Infection And Immunity

The goal of this program is to promote research on the interaction of malnutrition, infection and immunity. Specifically our interests focus on the modulating effect of specific nutrients on immune function and microbial virulence, on mechanisms of food allergies and immune response to ingested antigens, and on the interaction of nutrition and infection in the tropical environment and in American hospitals.

In FY '80 the NIAID program in Nutrition, Infection and Immunity consisted of 20 grants, a small portion of one contract, and one New Investigator Award. One intramural research project on Food Allergy was initiated. The extramural program was monitored by at least four project officers in the IAIDP and MIDP programs, in addition to the CSB.

During FY '80, Dr. Edelman continued his appointment as Vice-Chairman of the NIH Nutrition Coordination Committee (NCC). Through the NCC, Dr. Edelman helped to design and promote nutrition research programs at the NIH. His NCC activities included: 1) chairman of several Ad Hoc Study Sections to critique applications submitted to the Fogarty International Center for the support of national and international meetings; 2) NIH representative on the travel grant selection committee for the Western Hemisphere Nutrition Congress VI, held in Los Angeles, California in August, 1980; 3) member of an Ad Hoc Study Section of the NCI to review proposals submitted in response to an RFP on Nutrition and Cancer; 4) member of the Working Group on Lipids and Immune Function that met at the NCI-sponsored "Conference on Fat and Cancer" held in December, 1979.

Dr. Edelman participated in the "American Medical Association Working Conference on the Role of Single Nutrients in Immunocompetence" held in November, 1979 in Atlanta. He is co-editor of the conference proceedings to be published in the J.A.M.A. He continued as co-editor of the lengthy workshop proceedings of the NCC-sponsored "Workshop on Nutritional Support of the Patient: Research Directions for the 1980's." The proceedings will be published as a special supplement in The American J Clinical Nutrition in fiscal year 1981. Together with Dr. Lars A. Hanson, Fogarty Scholar-in-Residence, Dr. Edelman organized and co-moderated a meeting sponsored by the Fogarty International Center entitled, "A Discussion Group on Infections and Undernutrition" held in June, 1980. That meeting illustrated how infections retarded growth of children living in the Third World, and how individual nutrients and breast milk affected infections and immune function in these poor populations. A summary of the meeting will be published.

Dr. Edelman delivered an invited paper entitled "Obesity: Does It Modulate Infectious Diseases And Immunity?" at the "Symposium on Effect of Diet on Immunosuppression and Disease" at the Western Hemisphere Nutrition Congress VI, held in Los Angeles in August, 1980. An extended version of that talk will be published by the A.M.A. in the Proceedings of the Congress in FY 1981. The paper reviews the important, and newly discovered modulating effects of fatty acids and cholesterol on immune function, and suggests areas for future research.

Other Activities

Mrs. Mattheis collects and reviews the necessary documentation required to file and maintain Investigational New Drug Applications (INDAs) with the Food and Drug Administration (FDA) for the Microbiology and Infectious Diseases Program. Currently 20 active INDAs are filed with the Bureau of Biologics, FDA, and seven with the Bureau of Drugs, FDA. During the past year new INDAs were filed for "Live Varicella Virus Vaccine", "Inactivated Rocky Mountain Spotted Fever Vaccine", "Penicillin G, Sodium for Prevention of Group B Streptococcal Disease in the Newborn", "Ketoconazole", and "Parenteral Vidarabine, Vidarabine Monophosphate and Acyclovir". Staff support is provided for the NIAID-Clinical Research Subpanel which reviews both intramural and contract supported clinical studies.

Dr. Edelman delivered three invited lectures at the 27th Annual Convention of the Pediatric Section of the Puerto Rico Medical Association, San Juan in February, 1980. The three lectures were entitled, "Nutrition, Infection and Immunity", "Progress in Rapid Viral Diagnosis", and "Prospects on Antiviral Chemotherapy".

Dr. Edelman continued to serve on the following committees of other Federal agencies: 1) NIH liaison member of the Infection Control Committee of the Veteran's Administration, 2) the Human Ethics Committees (Institutional Review Boards) of the Walter Reed Army Institute of Research and the Frederick Cancer Research Center. As Clinical Associate Professor of Medicine, Dr. Edelman served as a preceptor for six weeks in Clinical Medicine, Uniform Services University of the Health Sciences.

Dr. Horton served as liaison member of the NIH Coordinating Committee on Cystic Fibrosis and participated at the Eighth International Congress on Cystic Fibrosis, May 26 - 30, 1980, Toronto, Canada. This highly productive conference provided an excellent exchange of scientific information to NIAID grantees and others working on pseudomonas infection and the control of this infection by prototype vaccines.

DEVELOPMENT AND APPLICATIONS BRANCH

Consistent with its name, the primary objective of the Development and Applications Branch is the translation of new information derived from basic research into methodologies appropriate for the control or prevention of infectious diseases in humans. The Branch is particularly involved in selected infectious disease areas designated as high priority for extramural support by the Institute. Fundamental and applied research activities include: identification of important infectious disease problems with potential for control or prevention through immunization or utilization of antivirals or hyperimmune sera, development of appropriate vaccines, antivirals, and other control measures, design and support of appropriate clinical trials for evaluation of control measures and basic research in related areas.

Approximate Level of Support

Activity	Number	Amount
Vaccine Evaluation Centers		
Research Contracts	7*	$ 1,310,538
Influenza		
Research Contracts	11	2,244,024
Interagency Agreements	1	69,786
Research Grants	22	2,186,436
Young Investigator Awards	2	85,500
Respiratory Diseases		
Interinstitute Agreements	1	53,874
Research Grants	6	459,357
Hepatitis		
Research Contracts	6	1,524,755
Interagency Agreements	2	87,390
Research Grants	8	804,879
Young Investigator Awards	1	42,879
Fellowships	1	18,380
Career Awards	1	37,584
Antiviral Substances		
Research Contracts	15*	1,016,197
Research Grants	33	2,456,817
Career Awards	2	74,672
Fellowships	3	43,180
Training Grants	2	134,040

Approximate Level of Support (cont.)

Activity	Number	Amount
Bacterial Vaccines		
Research Contracts	13*	$ 917,642
Research Grants	19	1,159,483
Career Awards	2	73,830
Fellowships	4	47,547
Viral Vaccines		
Research Contracts	1	256,389
Research Grants	6	417,730
Young Investigator Awards	1	27,655
Fellowships	1	13,400
Conference Support	1	21,250
Totals	172	$15,585,214

*There is some overlap in the number of contracts since there has been both expiration and initiation of contracts in this fiscal year.

Program Summary

The largest directed research component of the NIAID resides in the DAB. Areas designated as high priority include research in influenza, hepatitis, viral vaccines, bacterial vaccines, and antiviral agent development. The contract projects of the Institute have been the hardest hit by reductions in research funds, yet we have managed to maintain viable and progressive programs in these important areas.

Influenza continues to befuddle all attempts at prognostication. During the past year, most of the disease was due to influenza B; however, there was concurrent influenza A, both H1N1 and H3N2. Work on the development of a more effective vaccine continues to be emphasized. The importance of antivirals to supplement vaccines against this disease was stressed by a consensus meeting on the use of amantadine sponsored by the NIAID. Many observors indicated that this was one of the most thorough and successful consensus meetings sponsored by the NIH. One of the new areas of emphasis in this program has been Reye's Syndrome, a sequelae of viral disease, predominantly influenza. The Influenza Program has been designated as the focus of an NIH-wide coordinating committee on this subject.

Respiratory viral diseases, particularly those caused by respiratory syncytial and parainfluenza viruses, continue to be of major program concern, but progress remains slow due to the difficulty of working with these agents. One of the interesting leads is the role of the fusion protein in paramyxoviruses; it is hoped that this may prove to be a useful antigen for vaccine development.

Advances in the development of a useful vaccine against hepatitis B continue at a satisfying rate. Studies in hemodialysis patients are well under way, and studies on the vaccine prevention of neonatally acquired HBV are planned. The potential of the woodchuck as a useful model for HBV hepatitis and hepatoma is being pursued.

Interferon has received unprecedented publicity during the past year, largely through its discovery by the oncologists. The development of this agent is primarily due to the long-standing efforts of the NIAID Antiviral Substances Program. This has been recognized by the WHO through designation of this Program as the National Center for Interferon. The program continues to be attuned to new advances by expanding its efforts to immune interferon and cloning techniques. It has taken the lead by sponsoring interagency meetings, as well as international meetings (i.e., a meeting to establish a needed nomenclature scheme). During the past year, data from the study demonstrating the efficacy of adenine arabinoside against neonatal herpes were submitted for publication. New studies to evaluate adenine arabinoside monophosphate and acyclovir in herpes encephalitis and acyclovir against herpes labialis were initiated.

The Bacterial Vaccines Program is concentrating on the development of a multivalent meningitis vaccine and a vaccine for Rocky Mountain Spotted Fever. This program has been instrumental in the licensing of components of the proposed meningitis vaccine such as meningococcal Type A and Type C polysaccharides, and a multivalent pneumococcal vaccine. Currently it is also responsible for a large collaborative study to evaluate the pneumococcal vaccine in various high-risk populations such as sickle cell patients. These projects are yielding important information at little cost to the Institute. Epidemiological studies on Rocky Mountain Spotted Fever are progressing well, and vaccine efficacy studies in a closed facility are planned.

In FY80 the DAB was involved in the organization and conduct of six major meetings: Amantadine Consensus, US-USSR Collaborative Agreement on Influenza and Acute Respiratory Diseases, Interferon Testing (BoB/NCI/NIAID), Interferon Nomenclature, US-Japan Hepatitis Panel, Interferon Working Group, as well as several contractor and interagency meetings.

Research Highlights

Influenza Program

The last three years have been unprecedented in the history of influenza viruses. As did the two before it, last year continued to bring surprises. Viruses related to U/USSR/77 (H1N1) continued to be isolated during the 1979-80 season, although only sporadically in the United States. Surprisingly, genetic analyses of the H1N1 viruses revealed that they are not genetic reassortants with H3N2 viruses as had been observed in 1978-79. In addition, the isolation of other influenza A strains was reported, and a new antigenic variant identified, A/Bangkok/79 (H3N2). The epidemic of influenza that occurred was attributed to influenza B viruses. A new antigenic variant, B/Singapore/222/79, supplanted the previously predominant variant, B/Hong Kong/72. This epidemic was associated with excess mortality due to pneumonia/influenza, and represented only the second time the B viruses have been clearly associated with excess deaths since 1962. The year was also marked by a large number of cases of Reye's Syndrome. Although treatment and diagnosis have improved, this remains a devastating disease with a high incidence of mental retardation and other neurologic sequelae in survivors.

It is fortunate that excellent virologists and clinicians have been attracted to the field and many new observations have emerged during the last year. Among the more exciting developments have been the giant strides made in obtaining amino acid sequence data and three dimensional structure data on influenza hemagglutinin (HA). Regions of the molecule have been identified that play significant roles in viral penetration, viral exit from the cell and antigenic variation. Certain regions of the molecule have been found to be highly variable while others are remarkably constant. The amino acid sequence data have been compared with data obtained with monoclonal antibody, and cross-reactive and specific antigenic determinants are being identified. Three dimensional x-ray crystalographic data suggest that the HA molecule is a club-shaped trimer.

Recombinant DNA (RDNA) technology has also been applied to the analysis of influenza virus biology. For example, the technique has been exploited to sequence the HA gene and confirm the amino acid sequences obtained. These analyses have provided some of the evidence that covalently-linked signal peptides are required for the insertion of the HA into the cell membrane. In addition, data obtained from influenza virus genes cloned in E. coli have greatly enhanced our understanding of the nonstructural gene and of the source of the heterogenous sequences needed for priming viral RNA polymerase activities. The most intriguing aspect of this work has been the direct application of RDNA to harness the viral HA gene in bacterial cells so that HA can be produced more easily and in larger amounts. The HA genes of A/Victoria/75 (H3N2) and fowl plague virus, as well as others, have been cloned in E. coli and expression of these genes has been reported. This work, however, is still several years away from direct application in vaccine manufacture.

A workshop on Influenza B Viruses and Reye's Syndrome was held last year during which epidemiologic, genetic and molecular biologic aspects of influenza B viruses were reviewed along with the clinical manifestations of infection with these viruses. A summary of this workshop will be published in the September issue of the Journal of Infectious Diseases. In October, a consensus development conference on "The Role of Amantadine in the Treatment and Prevention of Influenza" was convened. The conference concluded that amantadine should be used because it has an important role in the treatment and prevention of influenza. The recommendations of the panel have received wide circulation and acceptance. Further studies are planned with amantadine to prevent transmission of influenza in family contacts, to reduce side effects with lower dosages, and to test rimantadine, an analogue of amantadine.

Programmatic emphasis during the last year has been placed on attenuated influenza vaccine development. Priority was given to testing both the cold-adapted (CA) and temperature-sensitive (ts) vaccines in a series of small closed studies to further characterize these candidates for genetic stability, reactogenicity and transmissibility. A ts vaccine (A/Hong Kong/123/77 (H1N1) ts1A2) was tested extensively in adults and found to be satisfactorily attenuated and stable. However, revertants in temperature-sensitivity appeared after administration to doubly seronegative children. Subsequent testing revealed that these revertants produced febrile illness in seronegative adults. As a consequence, further development of the tslA2 series of recombinants has stopped. The CA recombinants, however, have not shown any evidence of reversion, and further studies with these viruses are planned. The objective of these studies will be to test the efficacy of these vaccines against natural challenge. Work on the purified subunit vaccine has continued. Effort has been directed at potentiating antigenicity of these subunits by using liposomes or other acceptable adjuvants.

Currently available licensed, inactivated vaccines have also been tested. This work is a continuation of earlier efforts to investigate the possibility that exaggerated disease may occur in children who have been vaccinated. Over 400 children have been vaccinated and followed over the last four years. No evidence of exaggerated disease has been obtained in any of these children. These trials have been conducted with whole-virus and split-virus vaccines, and methods of administration of whole-virus vaccines to children under three years of age have been developed. Efforts have also been made to verify the observation of Hoskin's, et al (Lancet, January 6, 1979) that annual immunization of children with inactivated vaccines did not reduce cumulative attack rates for influenza after several years. The Influenza Research Center has started a study of the experience of heavily vaccinated populations, such as the elderly, with this in mind. A retrospective review of clinical records suggested that inactivated influenza vaccines prevented severe respiratory illness when compared to an unvaccinated cohort. However, for the present, these studies can only provide indirect evidence of efficacy since the studies are not prospective, not placebo-controlled, and have not included viral isolation data. They have, instead, attempted to correlate the clinical experience of vaccinated and unvaccinated individuals during known periods of influenza activity. Severe respiratory infections appear to be reduced by about 60 percent in the group receiving inactivated influenza vaccines.

Studies on the efficacy of inactivated vaccines in young children and college populations were also completed during the last year. At the University of Washington, efficacy of about 60 percent was observed in a college-age population during the recent B epidemic, even though these individuals had been vaccinated in 1978. Similar data were obtained in children.

Reye's Syndrome has been a major area of emphasis over the last year, one that was particularly severe with about 600 cases being reported to the CDC. A Reye's Syndrome Coordinating Committee was established at NIH with NIAID serving in the lead role. As a consequence of the Workshop on Influenza B and Reye's Syndrome and the current interest in Reye's Syndrome, a Program Announcement on Reye's Syndrome was published in collaboration with NINCDS, NIAMDD, and NICHHD. In addition, a Consensus Development Conference on the Diagnosis and Treatment of Reye's Syndrome has been scheduled for next March.

Viral Respiratory Diseases Program

The magnitude of infections and illnesses from parainfluenza viruses types 1, 2 and 3 and respiratory syncytial virus (RSV) is difficult to document, but estimates from several epidemiologic studies indicate that these viruses cause severe morbidity in pediatric populations. Recent evidence suggests that RSV can be associated with clinical illness in hospital support staff and in homes for the elderly. Of all severe lower respiratory diseases in infants and young children, perhaps 40 percent could be prevented if effective vaccines against these agents were available and used.

Successful immunization against these agents represents a formidable task. The immune state following natural infection, if existent, is short-lived and does not correlate with serum neutralizing antibody. RSV infection represents a serious threat to infants since the primary infection is associated with increased risk of lower respiratory disease. Efforts to develop attenuated RSV vaccines that will mimic natural infection, but not induce illness, have continued during the past year. One candidate, ts-2, was administered to 16 seropositive and 3 seronegative children, ages 12-15 months. No evidence of infection was detected, suggesting that the candidate is overattenuated. Administration to several seronegative infants, 6 months to 12 months of age will be required before final evaluation of this candidate can occur. Several other potential candidates, NG-1 and NG-16, are available for evaluation in children.

During the past year a CA RSV isolate developed by Sandoz, was evaluated in chimpanzees for levels of attenuation. This CA virus was not distinguishable from wild-type virus in the levels of rhinorrhea and virus titer produced, indicating lack of attenuation.

Biochemical and biophysical studies of RSV are being conducted in a number of good laboratories in the U.S. Recent evidence from NIAID grant-supported research indicates that the genome of RSV is single-stranded 52_S RNA, with negative strand sense. Previous evidence of DNA or a contaminating DNA virus in RSV preparations has been negated, and those observations have been attributed to technical problems during purification.

The role of fusion protein in paramyxovirus spread in tissue culture systems has provided impetus to identifying such a component in RSV. Evidence suggests that antibodies to the fusion protein may prevent infection or cell-to-cell spread of the virus in tissue culture. Evidence for such a protein in RSV is being sought by the Laboratory of Infectious Diseases (LID) and the Laboratory of Molecular Virology and Immunology (LMVI), Georgetown University. The LMVI has successfully developed hybridoma lines against RSV and is attempting to characterize the various viral antigens for which specific antibody is available. Studies with discriminating monoclonal antibodies during the next year should contribute to our science base in RSV.

The paucity of candidate vaccines for the respiratory viruses is a reflection of the difficulties in working with the viruses, lack of understanding of mechanisms of pathogenesis and immunity, and readily available animal models. However, the DAB is prepared to evaluate potential candidates developed within industry and academia, as well as Government laboratories, in animal models and, if warranted, in humans.

Viral Hepatitis Program

The goal of the Hepatitis Program is the control of hepatitis A (HAV), B (HBV), and non A-non B (NANB) infections. Because of its more serious clinical manifestations, HBV disease is of major public health importance in the U.S. Moreover, the global impact of the disease is more significant because it has been estimated that 176,000,000 carriers serve as a reservoir of infection. In addition, a close association between chronic HBV and primary hepatocellular carcinoma (PHC) has been demonstrated. PHC risk in Taiwan among chronic HBV carriers compared to non-carriers is approximately 230:1.

During the past year significant advances were made in developing and evaluating experimental hepatitis B vaccines. As reported previously, the adw vaccine prepared by the NIAID was not satisfactorily immunogenic in humans. Portions of the bulk vaccine were reprocessed to remove human serum albumin and prepared as aqueous antigen or alum-adsorbed vaccine(s). Although the aqueous vaccine was more immunogenic than the original product, the alum-adsorbed vaccine (AAV) was superior in terms of antibody conversion when tested in NIH volunteers by the LID. Approximately 75 individuals have received doses of 40, 20, 10 or 5 µg. The vaccine is administered in three doses--time 0, six weeks, and six months. Results indicate essentially no reactogenicity with approximately 80 percent of the vaccinees producing anti HB_s two to three weeks after the first inoculation with as little as 5 µg of antigen. Various parameters of immunization (i.e., sequencing of doses, local and systemic reactogenicity) are being investigated in larger numbers of volunteers in a placebo controlled trial at the Baylor College of Medicine. To date, the data indicate that the vaccine is essentially non-reactogenic and that there are seroconversion rates of 65 percent following the first dose of vaccine.

A vaccine efficacy trial conducted in hemodialysis patients and staff is well under way. This large trial, employing Merck adw vaccine, involves 29 hemodialysis centers, with anticipated enrollments of 1,000 patients and 900 staff members. The study encompasses two protocols--an initial immunogenicity trial in patients and the general placebo-controlled trial in staff and patients. The pre-trial immunogenicity study of vaccine in patients was designed to test (1) whether patients, many of whom are naturally immunosuppressed, would respond with anti-HB_s, and (2) if more than two injections at monthly intervals were required to stimulate anti-HB_s. After the trials were initiated, a third parameter, vaccine dose (i.e., 20 µg versus 40 µg), was introduced into the study. There was no significant difference in the percent responding among those receiving either two inoculations or three inoculations at one-month intervals. Seroconversion frequencies for 40 µg of antigen were approximately 70 percent at three months and exceeded 80 percent at seven months. The trial is being conducted with patients and staff receiving inoculation on the same schedule; staff members receive 20 µg of antigen and patients 40 µg of antigen per inoculation. To date, 749 patients and 568 staff have been randomized into the trial.

Another approach to prevention and control of HBV infection and sequelae is to interrupt maternal-fetal transmission. Neonatal acquisition of HBV infection is of major magnitude in parts of Africa and the Far East. Interruption of this route of transmission by passive immunization (HBIG) has been under investigation for the past 1½ years. Randomization into this placebo-controlled trial has been completed (282 babies), and it is anticipated that data will be evaluated in the Fall, 1980.

The prevention of neonatally-acquired HBV infection and its attendant long-term sequelae is one of the most important and exciting areas of biomedical research today. Whether this can be accomplished by vaccine, HBIG, or a combination of both, remains to be answered. Recent data from French studies being conducted in Senegal suggest that newborns are capable of mounting an antibody response to HBsAg antigen. However, trials need to be undertaken to fully evaluate responses to vaccine (reactogenicity, seroconversion rates, dosage and schedules), followed by an appropriately designed efficacy trial. Since such studies are presumed to entail post-exposure prophylaxis, the vaccine to be used should stimulate a rapid primary response. Populations for such studies have been thoroughly documented in Taiwan and are under investigation in the People's Republic of China and, possibly, Liberia. Prevention of early age acquistion of HBV infection and its sequelae may be the first documentable evidence of prevention by immunization of one type of human cancer.

Studies on the prevention of hepatoma by immunization may be approachable using the woodchuck (Marmota monax) model previously described. Collaborative efforts with NCI were begun last year with the establishment of a woodchuck breeding colony (Cornell University). Young animals of known serologic status are essential to the development of an animal model system for this virus. Another virus similar to human HBV has been recently discovered in the ground squirrel (Spermophilus beecheyi) by an NIAID grantee, Dr. William Robinson. The types of disease and sequelae caused by the virus, if any, remain to be defined.

Events during the past year with animal colony contracts supported jointly by the BoB/FDA, CDC, and NIAID have created programmatic concerns. Albany Medical College, contractor for the Holloman Air Force Base chimpanzee colony, has served notice to the PHS that it intends to divest itself of this activity. Plans are currently under way to find an alternate method to continue this extremely important facility.

The breeding colony for Saguinus mystax marmosets at the Delta Regional Primate Center (DRPC) recently suffered a viral outbreak that decimated the original colony. There is continuing need for marmosets susceptible to HAV, and the justification for establishing a breeding colony is still appropriate. Therefore, the BoB/FDA is currently readvertising this project. The scope of work will be modified to include S. labiatus marmosets which appear to be highly susceptible to HAV infection and are hardier in a breeding colony environment. It should be noted that no laboratory has been successful in breeding S. mystax in large numbers.

Recently, a newly recognized antigen specificity, delta antigen (δ) has been partially characterized by LMVI and NIAID's intramural scientists. This antigen, first detected by immunofluorescence in the livers of human subjects with chronic HBV infection, appears to be an internal component of the 37 nm subpopulation of HBsAg. An RNA species (5×10^5 MW) was temporally associated with δ in sera and co-purified with the δ-associated subpopulation of HBsAg. The inoculation of sera from patients with markers into HBV susceptible chimpanzees resulted in HBV hepatitis and markers in recipient animals. The significance of this new antigen (new agent?) is currently under intensive investigation.

Treatment of the chronic carrier with drugs is under active investigation with encouraging results (see Antivirals section).

HAV remains a problem in sporadic outbreaks, and due to shifting patterns of susceptibility, poses a threat to travelers to geographic areas of high endemicity. Breakthrough in the cultivation of HAV lends promise to future vaccine development efforts. Finally, NANB hepatitis accounts for high morbidity in sporadic hepatitis and following blood transfusions. Long-term sequelae are difficult to assess at the present time, but chronicity has been well established. Breakthroughs in this area have been slow, but it appears to be only a matter of time before assays for virus(es) and antibody(ies) are developed. Once this essential barrier is overcome, progress will be rapid and insights into therapy and prevention will be forthcoming.

The First Joint US-Japan Hepatitis Panel Meeting was held in New York City in March, 1980. A summary of the meeting will be published in the Journal of Infectious Diseases.

Antiviral Substances Program

Viral diseases are leading causes of morbidity and mortality throughout the world, and the use of antivirals is an important means of treating and preventing these illnesses. Although development of host resistance remains the first line of defense, there are many viral diseases for which vaccines are not available or cannot be given to all people at risk. Thus, chemotherapeutic agents with low toxicity and high efficacy are needed. The support, encouragement, and coordination of antiviral research through the Antiviral Substances Program (ASP) has made treatment with antiviral drugs a reality for some diseases, including herpes encephalitis and influenza A, and an important prospect for other infectious diseases and cancer.

The ASP identifies both chemical and biological antiviral drugs that hold particular promise for an infectious disease. If preclinical studies show that the compound has high efficacy and low toxicity, clinical trials may be undertaken. In order to initiate clinical trials, adequate toxicity, teratogenicity, and mutagenicity data must be available. The FDA has strict requirements for these data before an IND is approved. The Program does not have a formal mechanism for obtaining this information but works with the drug companies to obtain the necessary data. Once all the data are available on selected drugs, carefully focused clinical trials are supported by the Program.

The ASP maintains a group of animal model systems, closely mimicking human disease, to rapidly evaluate promising antiviral compounds. These animal model contracts, therefore, serve a very important function for the Program. In an effort to both maintain competition and the most effective contracts, three of these projects were readvertised. Thirteen proposals were received, attesting to the expanding interest in antiviral research. Through these contracts, optimal methods and routes of drug administration are studied. Two new compounds that have been shown to hold particular promise for treatment of herpes infections are acyclovir (ACV) and adenine arabinoside monophosphate (ara-AMP). Based on these animal model studies, the NIAID is currently testing their clinical efficacy.

Studies to date have demonstrated the effectiveness of adenine arabinoside (ara-A) for HSE, resulting in licensure of the drug in October, 1978. Follow-up studies in 75 cases of HSE confirmed the initial findings that ara-A reduced mortality to 40 percent in treated patients versus 70 percent in untreated patients. Mortality rates were lowest in individuals lethargic at the time of diagnosis (22 percent) versus those in the semi-comatose or comatose state (55 percent); these data are being prepared for publication. There is still a need for further improvement in drug therapy for herpes encephalitis. Because of the low solubility of ara-A, it is a difficult drug to administer; currently, patients receive a 12-hour infusion. Based on the animal model studies which suggested that acyclovir and ara-AMP were at least as effective as ara-A, a new HSE protocol was begun comparing ara-A, ara-AMP, and ACV. In addition, since ara-A was shown to be efficacious for neonatal herpes, a study employing a higher drug dosage is under way in an attempt to enhance therapeutic effect. A manuscript presenting data on neonatal herpes infections has been submitted for publication, and it is expected that FDA approval for use of this drug against this infection will be forthcoming.

It is estimated that 50 percent of the adult population suffers from herpes labialis, and trials are under way to determine if topical ACV alters the disease course. Results from the initial clinical trial are encouraging; ACV significantly reduced the replication of the virus in the treated lesions. Based on these findings, a follow-up clinical trial is being planned with treatment to be initiated earlier in the course of the disease and with more frequent drug application. These findings on the topical administration of drugs can be applied to the treatment of similar infections, such as genital herpes.

Other clinical studies in progress include phase one studies with chronic hepatitis B infection. Though human leukocyte interferon and ara-A may alter the course of a small percentage of chronic hepatitis B disease, the most promising regimen appears to be a combination of these two drugs. Following multiple cycles of combined interferon and ara-A, sera from 7 out of 16 patients showed permanent loss of detectable DNA polymerase activity. Controlled clinical trials in hepatitis B are planned to determine the most promising drug regimen.

Interferon is a broad spectrum antiviral substance effective against RNA and DNA viruses; support of research on this natural product is an important component of the ASP. One of the major components of this program has been the development of standards for human leukocyte interferon, human fibroblast interferon, rabbit interferon, and mouse interferon. In addition, antisera to human and mouse interferon are available and have been instrumental in the characterization and identification of interferon specific reactions. The Program has also developed fibroblast cells suitable for production of interferon. Recent studies have indicated that there are several types of interferon generally distinguished by their antigenic type. The classical "type one" interferons have a high antiviral activity and are acid stable in contrast to "type two" interferons which have, in addition to antiviral activity, a relatively powerful effect on the immune system and are acid labile. The NIAID is currently producing new standard reagents for "type two" mouse and human interferons as well as antisera to these interferons. These reagents will become NIH standards available to scientists throughout the world. After considerable experience is gained with these reagents, the WHO is prepared to adopt them as the international standards.

The NIAID sponsors workshops in important areas of interferon research. During the past year, the NIAID supported a New York Academy of Sciences' Workshop entitled, "Conference on Regulatory Functions of Interferons." The NIAID also sponsored a workshop on interferon nomenclature. Interest in interferons in recent years has greatly increased, and now immunologists, cell biologists and cancer researchers have discovered interferon. It is, therefore, necessary to devise a universally accepted nomenclature system. The suggested system is as follows: alpha - for interferon with leukocyte antigenic specificity; beta - for interferon with fibroblast antigenic specificity; and gamma - for the type 2 or immune interferons. This nomenclature system will be further discussed at the International Interferon Conference to be held in April, 1981, in The Netherlands.

An important part of the NIAID interferon effort continues to be the fostering of exchange of information among the NIH institutes, and several meetings have been organized by NIAID for this purpose. The NCI is providing funds for the two new contracts to make the new immune interferon standards and antisera. The NIAID began an NIH cooperative study to develop procedures for production of interferon by recombinant DNA technology. It is expected that these studies will add useful information to the rapidly expanding area of interferon cloning. The NIAID and the WHO jointly sponsored a workshop in September, 1980, that brought together scientists working on developing recombinant DNA technology for interferon production to foster exchange of information and cooperation in this highly competitive and complex field.

Improvement in the understanding of viral replication may permit the development of targeted antiviral compounds. The Program has been able to initiate a pilot effort in targeted antiviral drug development based on our knowledge of the viral nucleic acid sequence. Influenza A is being used as the model virus, and nucleic acid sequences that will interfere with and inhibit the replication of viral RNA and mRNA are being synthesized.

Other exciting results have come through the grant support of antiviral research. Clinical trials have shown that exogenous interferon prevents reactivation of herpes infection after neurosurgery. In other studies, ara-A action is being evaluated against cytomegalovirus infections, particularly chorioretinitis. These initial experiments have shown that ara-A decereases inflammation and virus shedding and improves retinitis.

Many grants are involved with the study of the basic nature and mechanism of action of interferon. Recent studies have shown that one type of mouse interferon is antigenically related to human interferon, suggesting that interferon molecules from different species are similar. Other work shows that interferon reduces 2', 5' oligopolymerase and protein kinase, and that these enzymes are involved in preventing virus replication. Work on new and better ways to produce interferon is being supported, and studies have shown that macrophages can produce interferon. These basic research studies serve as the foundation for the targeted research programs necessary to improve the treatment of many important viral infections.

Bacterial Vaccines Program

Pneumococcal Diseases: Studies sponsored by the NIAID during the past 13 years, along with studies by manufacturers, have led to the development and licensure of a 14-valent pneumococcal polysaccharide vaccine. The vaccine was licensed based on efficacy data for prevention of pneumococcal bacteremia and pneumonia; however, it may be useful in patients with various disease conditions that predispose to pneumococcal infections. These include persons with sickle cell disease, Hodgkin's disease, diabetes and various cancers, as well as renal dialysis and transplant patients. There are approximately 40 ongoing studies to determine effectiveness in these populations. Although the investigators receive no direct funding from NIAID, the protocols are being coordinated by staff, and all antibody assays are being done through the contract-supported facility at SUNY, Downstate Medical Center. Initial results suggest: (1) Sickle cell and splenectomy patients appear to respond well to the vaccine; (2) Hodgkin's patients who have undergone splenectomy and chemotherapy respond poorly to vaccine; however, Hodgkin's patients given vaccine prior to therapy appear to respond normally; (3) antibody response is diminished in renal dialysis patients; (4) simultaneous administration of pneumococcal and influenza vaccines does not have a negative effect on response to the individual antigens; (5) diabetics and patients with nephrotic syndrome appear to respond normally to vaccine; (6) systemic lupus erythematosus patients respond normally to the vaccine without detectable alterations of the underlying disease; and (7) preliminary data indicate that patients with chronic obstructive pulmonary disease also respond well. When all the collaborative studies are completed, it may be possible to make further recommendations on the use of pneumococcal vaccine.

Pneumococci are estimated to be responsible for 40 to 50 percent of acute otitis media in children, often causing hearing disorders and subsequent learning disabilities. A program to study the value of pneumococcal polysaccharide vaccine for the prevention of otitis media was initiated in 1975. Two double-blind efficacy trials, at Boston City Hospital and Huntsville (Alabama) hospital, of an octavalent vaccine for prevention of disease in children who have had previous middle ear infections have recently been completed. Preliminary data from the Huntsville study indicate that pneumococcal otitis media due to types in the vaccine may be reduced as much as 50 percent by use of the vaccine. Preliminary data from Boston do not show significant protection by the vaccine; this picture may change upon final analysis of the data. A third project at Vanderbilt University was designed to study dose and booster regimens in infants. The response of infants to the polysaccharides follows three modes. Response to type 3 polysaccharide is quite good with significant antibody levels at age six months. Other types such as 7, 18, and 23 cause good responses at 12 months; types 1, 6, 14 and 19 were poorly immunogenic even at 12 months.

An animal model has been developed to study pneumococcal otitis media. Using the chinchilla, the investigators have been able consistently to establish otitis media with intranasally administered pneumococci when negative pressure is applied to the middle ear. They have also been able to establish pneumococcal otitis media with intranasal inoculation, and without the negative pressure, if influenza virus is simultaneously administered. Vaccination can be correlated with protection from disease in this model, even in cases in which there has been no measurable serum antibody response to the polysaccharides. This has important implications for the human efficacy trials.

Grant supported research includes studies on the role of the complement system in the host's defense against bacterial infections. A subcellular component of the pneumococcus (teichoic acid) has been shown to be capable of activating the alternative complement pathway.

Meningitis: It is estimated that approximately 20,000 cases of bacterial meningitis occur in the U.S. each year, mostly in young children and infants. A pure polysaccharide vaccine for H. influenzae type b, developed and tested under our program, is ineffective in children under 18 months of age. Because most of the H. influenzae meningitis occurs in this younger age group, program efforts are now directed toward developing vaccines effective in infants. A new H. influenzae vaccine which is a complex of polyribose phosphate (PRP) and cell wall material, primarily polypeptides and lipopolysaccharide, is under study. Designated PRPc, this material has been tested in adults and children. Although studies are continuing in small children, results have not been encouraging. The following alternative approaches to preparing an improved H. influenzae vaccine are currently being pursued: (1) extraction and characterization of outer membrane proteins from H. influenzae to determine their role in disease and potential as immunogens; (2) preparation of a complex of PRP with a pure protein such as diptheria toxoid; (3) preparation of complexes of highly immuogenic bacterial polysaccharides with determinant groups of PRP; and (4) preparation of very high molecular weight molecules of PRP.

In addition to efforts to improve the infant's response to bacterial meningitis vaccines, protection of the neonate and young infant by hyperimmunization of the mother is being attempted. H. influenzae PRP was given to women who planned pregnancies and to a control group of women who did not plan to become pregnant. Preliminary data show that in those women who responded well to the vaccine, the level of antibody in the cord blood and the infant was comparable to the mother's antibody level. Antibody levels in the infants will be followed to see how long they last and whether there is interference with the child's later ability to respond to the vaccine. Unfortunately, some women did not respond well to the vaccine so there were only low levels of antibody in their offspring.

Groups A and C meningococcal polysaccharide vaccines have been licensed as a result of efficacy studies in adults and older children. The group A vaccine has been shown to be effective in children down to three months of age in an efficacy trial in Finland sponsored by the NIAID. Group C meningogoccal polysaccharide vaccine is poorly immunogenic in very young children. A capsular polysaccharide extracted from a variant group C strain has been prepared at Rockefeller University. This new material is of a different chemical structure than the licensed group C vaccine, but it is antigenically cross-reactive. Through the NIAID program, this variant C vaccine has been shown to be superior to the licensed material in adults and children over two years of age. Studies in infants are under way at the present time.

Group B meningococcal polysaccharide is non-antigenic in humans. A candidate protein vaccine, extracted from the outer membrane layer of the organism, has been developed by the Bureau of Biologics/FDA. Phase I safety and antigenicity tests in adults and older children show that these age groups do respond to the vaccine but the response is disappointing. Studies are beginning in infants, however, as there are reasons to believe that children may respond better than older individuals. A second vaccine, a combination of outer membrane protein and a high molecular weight group B polysaccharide, has also been developed by the BoB. Evaluation of this material in adults is beginning.

There are a limited number of grants in the bacterial meningitis area. One grantee is beginning studies on the role of secretory IgA in the host's defense against meningococcal infections, and several grantees are looking at the cell envelope proteins of H. influenzae as possible infant immunogens. Program staff is encouraging all efforts toward the development of meningitis vaccines for use in infants in view of the fact that most of the pure polysaccharides have been non-immunogenic under 18 months of age. Efforts are being made to encourage research either toward modifying vaccines or better understanding the infant immune system so that we can overcome the lack of response in this age group. Toward this goal, a Program Project Grant RFA was developed during the past year for the establishment of "Research Unit(s) for the Prevention of Infectious Diseases in Infants." These units would have concentrated on developing vaccines or procedures for protection of the infant. Unfortunately, because of fund limitations, no awards could be made; however, one R01 award to study the infant immune response to H. influenzae antigens was made in FY 1980 and thus will represent the initial effort of the Program in this direction.

Rocky Mountain Spotted Fever: The presently licensed vaccine for RMSF is not considered effective and is no longer commercially available. A new, inactivated, whole-cell vaccine has been developed at the U.S. Army Medical Research Institute for Infectious Diseases (USAMRIID). Because of our mutual interest, the NIAID has assisted with support of phase I clinical trials of the vaccine in adults at the USAMRIID facilities at Fort Detrick. The vaccine protects animals and is immunogenic in man; additional clinical studies are being planned by NIAID. To locate potential populations for future vaccine trials, the NIAID has recently initiated two studies of the epidemiology of RMSF in North Carolina and Maryland. Another contract to further study the USAMRIID vaccine in a guinea pig model is making progress; guinea pig breeding to develop a strain with consistent susceptibility, production of purified antigens for comparison with vaccine antigens, and assessment of local vaccine effects are under way.

Pertussis: Pertussis is an area of considerable interest to program staff because the currently licensed vaccine, while effective, is sometimes responsible for rather serious reactions. Considering the advances that have been made in the past 10-15 years in the development of subcellular bacterial vaccines, it would seem that the technology is such that an improved pertussis vaccine is feasible. There are currently two grants in this area in the Bacterial Vaccines Program; one grantee has made considerable progress in defining the nutritional requirements for maintaining strains of *Bordetella pertussis* with stable antigenic mosaics, and is attempting isolation of components of the cell which may be useful potential vaccines. A second grant awarded in this fiscal year will also concentrate on the examination of various cellular components for their role in pathogenesis and their potential as candidate new vaccine antigens.

Viral Vaccines Program

The viral vaccine program covers those areas where research should be stimulated toward the goal of an effective vaccine but that are not covered by the other specific programs of the Branch. As a result of several workshops, it became clear that an attenuated varicella vaccine developed in Japan showed promise and should be field tested in the U.S. A contract with NYU to evaluate the vaccine in immunosuppressed children has been initiated. The first phase is to calibrate the titer, reactogenicity and antigenicity of the candidate vaccine in adults prior to using it in children. The Institute also has a grant with the University of Texas for similar phase 1 studies. Because of the difficulty in working with varicella and the need to determine markers of attenuation, several related grantees are working in conjunction with this overall vaccine development attempt. The program is further interested in research on the antigenic structure and other characteristics of the herpes viruses in the hope of developing more effective vaccine candidates not only for varicella, but for herpes infections in general and genital herpes in particular.

The Development and Applications Branch also supports five General Vaccine Evaluation Units that form the backbone of the Branch's efforts for the control of important disease problems. Through these groups, different populations, including all age groups, normal and "at risk," are available. These groups are utilized for the evaluation of all vaccines, viral and bacterial, and antivirals of interest to the Branch. During this past year these Units have been overtaxed and a backlog of work has developed.

EPIDEMIOLOGY AND BIOMETRY BRANCH

The Branch expanded its activities in research with the initiation of several projects by Dr. Richard A. Kaslow and in extramural activities with the acquisition of a major portion of the enteric disease program.

Approximate Level of Support

Activity	Number	Amount
Infectious Disease Epidemiology		
Research Contracts	2	182,948
Research Grants	3	429,631
Fellowships	1	16,118
Diarrheal Diseases		
Research Contracts	6	749,029
Research Grants	23	1,502,707
Program Projects	1	432,744
Fellowships	3	45,932
Training Grants	1	36,504
Totals	40	3,395,613

Epidemiologic and Biometric Consultation

In the area of data monitoring for clinical trials, Dr. William Blackwelder assisted contractors studying abbreviated courses of therapy of amphotericin B and 5-fluorcytosine for cryptococcal meningitis, efficacy of prophylactic penicillin for neonatal infections with group B Streptococcus, and efficacy of antiviral treatment of herpes labialis. As the Institute supports more collaborative clinical trials, these activities will grow as statistical advice is more frequently required in both study design and data monitoring.

Dr. Blackwelder concluded the development of methods to facilitate more precise calculation of pneumococcal antibody determined by RIA in the laboratory of Dr. Gerald Schiffman. The Branch staff provided statistical consultation to investigators studying pneumococcal vaccine in otitis media and lupus erthyematosus, assisted in managing a large volume of epidemiologic and clinical data generated by one enteric disease center, collaborated with Dr. David Alling, LCI, NIAID and Sir Charles Stuart-Harris, Fogarty Scholar, in analyzing national influenza and pneumonia data to refine epidemiological association of increased mortality rates and major activity of influenza in the U.S., and assisted investigators at the Walter Reed Army Institute of Research in designing a multicenter study to determine the value of an IgE standard developed by the IAIDP and in analyzing the resulting data.

Trans-NIH Activities

Members of the Branch Staff participated in many trans-NIH and Non-NIH committees. Dr. Blackwelder was active in a research advisory group which was convened by the Cystic Fibrosis Foundation and charged with developing a protocol for a multicenter clinical trial which might be sponsored by the agency. Dr. Curlin represented the Institute in the Digestive Disease Coordinating Committee and Dr. Kaslow functioned as the Institute's representative to the NIH and PHS Diabetes Committees. Dr. Curlin and Dr. Kaslow both were active in the NIH Epidemiology Committee.

Epidemiologic Projects

Dr. Richard A. Kaslow, research epidemiologist in the Branch, instituted a collaborative field study of Lyme disease in Wisconsin with Dr. Jeffrey Davis, Wisconsin Department of Health, and Dr. Andrew Spielman, Harvard University School of Public Health. Initial findings suggest the disease is underreported and is localized in areas in which the supected tick vector is found. An effort was begun to increase physician awareness to detect additional cases so definitive epidemiologic studies can be initiated. Epidemiologists in the Branch initiated with Dr. Daniel Mullally of the Asthma and Allergic Disease Branch, IAIDP, and Dr. William Howard of the Children's Hospital National Medical Center a 20 year retrospective analysis of admissions to the Hospital for asthma. A thorough methodological review was undertaken first to document the increase in asthma admissions suggested by Dr. Howard's preliminary tabulations and then to search for factors that could account for the increase.

HLA and Epidemiology

A collaborative study of lupus erythematosus in black Americans was initiated with the scientists of the NCI and investigators at the Johns Hopkins School of Medicine. A questionnaire developed by Dr. Kaslow and Dr. Jill B. Schiff will be administered to cases and carefully selected controls to seek possible environmental risk factors, and the HLA type of both cases and controls will be determined. This study seeks to develop an epidemiologic methodology in addition to identifying HLA associations with this disease in the black population. Dr. Kaslow and colleagues at the CDC and Stanford University prepared to expand epidemiologic studies in victims of common source enteric disease epidemics, to define further the extent to which Reiter's syndrome is associated with specific bacterial agents, and to identify immunogenetic charateristics that regulate development of this immunologic disease. Dr. Kaslow's previous work at the CDC identified probable Reiter's syndrome among patients affected in _Shigella flexneri_ epidemics but failed to identify Reiter's syndrome among patients of _Shigella sonnei_ outbreaks. Pursuit of these observations may identify specific bacterial antigens which trigger the pathologic immune response in patients with the HLA-B27 phenotype.

Field work began on the contract to Johns Hopkins University to note the influence of HLA in the immune response to viral vaccines in humans. Interviews were conducted and baseline sera were obtained in the Amish population. Children of all ages from a large proportion of families studied were found to be sero-negative to rubella. Thus, it will be quite feasible to enroll a sufficient number of volunteers to conduct the study. The Amish population will benefit more than anticipated from the vaccinations offered by the study.

Epidemiology of Nosocomial Infections

Support for the CDC SENIC project concluded, and a grant to study the epidemiology of nosocomial infections was awarded to the Rhode Island Department of Health. This ambitious effort, which utilizes a sample of patients drawn from all general hospitals in the state, will identify host factors modifying efficacy of infection control programs.

Enteric Diseases Centers

The initial three-year phase was completed at the three incumbent Infectious Enteric Disease Study Centers. Many of the findings which were previewed in the previous report were confirmed or extended. At all three centers rotavirus was the leading cause of diarrhea, and similar epidemiological patterns of this disease were observed. Rotavirus infections were more common in children with the highest rates observed in those between 6 to 18 months of age, and rotavirus diarrhea was also more serious than other diarrheal illness. Children in a lower socio-economic environment were first infected with rotavirus at an earlier age than those living in a more advantaged situation, although experience with rotavirus was virtually universal by the end of childhood. Reinfection with rotavirus was not uncommon. Reinfection in early childhood was likely to be caused by a different serotype, adults acquired the disease despite pre-existing antibody, and 10-20% of the hospital staff caring for rotavirus cases became infected even though the prevalence of antibody was high in this young adult age group. In 44% of the families with a rotavirus-infected infant, an older sibling or adult in the family was also infected, but reinfections in adults were generally asyptomatic or associated with only mild diarrhea. The role of persons with asymptomatic infection or mild diarrhea in the transmission of rotavirus diarrhea remains to be identified.

Diarrhea was a common problem in day-care centers (DCC), where infections with Shigella spp., G. lamblia and rotavirus were seen. Most cases occurred in outbreaks during which one half of the children in the DCC were ill, and children who acquired diarrhea at DCC frequently transmitted the infection to their families. The mode of transmission has not been identified, although evidence suggests person-to-person transmission may be important. In pediatric nurseries, transmission occurred among babies hospitalized in open wards, but babies in isolation apparently were less likely to contract the disease.

Enteropathogenic and enterotoxigenic E. coli accounted for less that 3% of enteric pathogens identified, and the newer bacterial and viral agents of diarrhea in which an etiology was determined was disappointingly low. It is clear from study of outbreaks and many thoroughly investigated isolated cases that a significant amount of diarrhea may be caused by undiscovered microbial agents or by non-infectious factors. Overall diarrheal morbidity rates appeared to be less age-related than presumed previously. The diarrhea rate among children under 1 year of age was 1.01/per year compared to an adult rate of 0.43/per year, but medical attention was not sought as frequently for diarrhea in adults. Work-time lost caring for children was the major cost for treatment of diarrhea.

Cholera

Nontoxigenic O-1 V. cholerae continue to be isolated from the environment and from fresh shellfish harvested from the Gulf Coast, but the public health significance of these isolates is unclear. Although V. cholerae isolated from brackish water contain genetic material required to make toxin, they do not produce toxin which can be detected by several very sensitive assays. Environmental strains from Bangladesh, by contrast, are toxigenic. Investigators are searching for other virulence factors in toxigenic strains which have been associated with clinical disease.

Vaccine Development

Investigations of mucosal immunity demonstrated a mechanism whereby distant encounter with antigen evokes an increased concentration of IgA in the bowel in an animal primed with small bowel exposure to the antigen. Although the response is less dramatic than intraluminal boosting, these findings appear highly relevant to efforts to develop local immunization for cholera and for other enterotoxigenic diarrheas. Oral administration of two 63 mg doses of crude toxin produced protection against lethal cholera challenge in the dog model, but purified toxin or B subunit did not produce equally high levels of protection. This raises the possibility that antigens other than toxin play a role in inducing protection.

The long-awaited field trial of a combined whole cell cholera vaccine and cholera toxoid was dropped from consideration because scant amounts of somatic antigen were found to contaminate the toxoid component. This may have precluded a definitive interpretation of any possible synergistic effect. Meanwhile other experiments showed a synergistic effect of various cell-wall and enterotoxin materials. Future consideration of a trial to test for synergy between anti-bacterial and anti-toxin immunity appears justified. Two non-toxigenic laboratory-derived mutants of V. cholerae were characterized and tested in human volunteers. A summary of this research and other diarrheal disease studies in the Center for Vaccine Development at the University of Maryland is contained in the report of the Clinical Studies Branch, MIDP.

E. Coli Diarrhea

E.coli diarrhea remains a significant health problem in many areas of the world. It is the leading cause of diarrhea among children in developing countries, although rotavirus is the most commonly identified pathogen among hospitalized infants, and approximately 50% of Americans traveling in developing countries acquire the disease. Investigators in several laboratories continued to study pili and colonization factors. These materials have been shown to be immunogenic in human volunteers especially when purified antigens were administered orally rather than parenterally.

MOLECULAR MICROBIOLOGY AND PARASITOLOGY BRANCH

The Molecular Microbiology and Parasitology Branch plans and conducts research grant, program project grant, contract, training grant, fellowship and career award programs in molecular microbiology, biochemistry, genetics, DNA recombinants, physiology, parasitology and medical entomology. It also coordinates the activities of the Parasitic Diseases Panel of the U.S.-Japan Cooperative Medical Science Program.

Appropriate Level of Support

Molecular Microbiology Section

Activity	Number	Amount
Research Grants 1/	235	$16,023,066
Research Contracts	1	337,903
Training Grants	4	312,168
Fellowships	38	527,964
Sub-total	278	$17,201,101

1/ Includes 8 Career Awards ($281,227)

Parasitology and Medical Entomology Section

Research Grants 2/	201	$12,856,666
Program Project Grants	2	832,395
Research Contracts	3	291,882
Training Grants	9	567,207
Fellowships	19	266,552
Sub-total	234	$14,814,702

2/ Includes 6 Career Awards ($213,356)

Branch	Number	Amount
Research Grants 3/	436	$28,879,732
Program Project Grants	2	832,395
Research Contracts	4	629,785
Training Grants	13	879,375
Fellowships	57	794,516
TOTAL	512	$32,015,803

3/ Includes 14 Career Awards ($494,583)

Molecular Microbiology

During FY 1980 a number of domestic meetings pertinent to the research supported by the Section were attended.

In early October, 1979, Dr. Delappe attended a meeting of the Mid-Atlantic Extrachromosomal Elements Group at Virginia Beach, Virginia where he addressed the group on programs relevant to the interests of its members; mainly our interests and goals in support of plasmid research and recombinant DNA. In late September, 1980, he will attend the annual meeting of the same group, this time at the Boar's Head Inn near Charlottesville, Virginia.

The section was represented at the Gordon Conference on Extrachromosomal Elements held in Plymouth, New Hampshire, in June.

The Branch ran a workshop in late April on the Application of the Recombinant DNA Technology to Protozoology. The meeting, to our knowledge, was one of the first, if not the first, of its kind to be held. Molecular biologists, protozoologists, and scientists already applying the technology to protozoology were brought together in an attempt to achieve a process of cross-fertilization. The meeting was well received by those who attended.

An invitation has been extended to Dr. Delappe to address a Conference on the Present Status and Future of Parasitology, sponsored jointly by the Rockefeller Foundation and the Josiah Macy, Jr. Foundation, at the Hotel Maison Dupuy, New Orleans, Louisiana. The conference will take place October 20-22, 1980.

Two of our grantees received honors during the year: Dr. Donald Helinski (University of California-San Diego) was elected to the National Academy of Sciences, and Dr. Robert Burris (University of Wisconsin) received a Presidential Science Medal. The former works with plasmids, the latter, nitrogen fixation.

Dr. Julian Davies will take a two year leave of absence from the University of Wisconsin during which he will function as Director of Research for Biogen, a molecular cloning company, in Geneva, Switzerland.

Program Summary

In addition to the basic free-ranging research of relevance to the Institute, there are two structured programs supported by this section of the Branch, one of which involves mechanisms of resistance to antimicrobial agents and the other, recombinant DNA.

(1) Mechanisms of Resistance to Antimicrobial Agents

During the past ten years, the problem of microbial resistance to therapeutic agents has become increasingly apparent. The relevance of this phenomenon to other interests of this Institute such as hospital-associated infections due to staphylococci, gram negative bacteria, and other organisms, together with investigations of gonococci and pneumococci, is obvious.

There is substantial clinical and epidemiological evidence that development of antibiotic resistance, and especially plasmid (extrachromosomal) mediated drug resistance, represents a growing problem in medical care. Most of the projects are concerned with defining the molecular and biochemical basis for resistance, the principal goal being to elucidate the fundamental biological mechanisms involved in the development of drug resistance by microorganisms. Specific goals involve investigation of the origin, development, evolution, expression and mechanism of drug resistance in a variety of specific microorganisms. Examples of microorganisms of particular interest are (but not limited to): Haemophilus influenzae, Enterobacteriaceae, Pseudomonas, Neisseria, staphylococci, streptococci, mycobacteria, mycoplasmas, and pathogenic fungi.

Research Highlights

AI 07168-14 B. Stocker (Stanford Medical School): Dr. Stocker has found that plasmid R46 (an R factor conferring resistance to ampicillin, sulfonamides, streptomycin, and tetracycline) reduces the bactericidal effect of ultraviolet irradiation but increases its mutagenic effect and raises the frequency of spontaneous reversion. Putative deletion mutants of R46 were obtained by transduction of the plasmid, then two successive conjugal transfers. Plasmids of five or six deletion classes, each with a different combination of drug resistance traits, retained conjugative ability and the UV-protecting, mutagenesis-enhancing and mutator effects of R46. Plasmids of a sixth class, represented by pKM115, conferred resistance only to streptomycin and were non-conjugative. All of several such plasmids (of independent origin) had a much stronger mutator effect than did R46, but lacked UV-protecting ability and did not enhance the mutagenic effect of UV irradiation. It is inferred that R46 possesses: (1) a gene, uvp, which increases capacity for error-prone repair of UV-damaged DNA, and thus causes both UV protection and enhancement of UV mutagenesis; (2) gene(s) whose action in the absence of gene uvp greatly increase the frequency of spontaneous reversion. A plasmid of another incompatibility group, pLS51, has UV-protecting and mutagenesis-enhancing effect but lacks the mutator property; introduction of pLS51 into a clone of hisG46 carrying a pKM115-type plasmid greatly reduced its spontaneous reversion rate, as expected if pLS51 also has a uvp gene able to modulate the mutator effect of R46-derived gene(s) in the pKM115-type plasmid. The above plasmids are found in Salmonella typhimurium.

AI 15189-04 M. Syvanen (Harvard Medical School): Bacterial mutagenesis using the transposable drug resistance elements is proving to be a very powerful tool in the genetic analysis of bacteria and their associated plasmids; this follows from the ability of these elements to insert into a very large number of bacterial and plasmid regions, including structural genes. Many different insertion mutations that inactivate bacterial genes have been obtained in this manner. The second property which makes these elements so useful in genetic analysis is that the insertion can convert an antibiotic sensitive bacteria into one that is now resistant to the respective antibiotic. Hence, one can monitor the insertion directly by resistance to the antibiotic independently of the phenotype caused by inactivation of the bacterial gene. This is particularly useful in mapping genes whose complete inactivation does not affect cell growth and in the analysis of recessive-lethal insertion mutations.

Insertions of Tn903, a transposable kanamycin resistance element, in bacteriophage lambda at a site to the right of gene R at 0.95 on the lambda physical map adversely effect growth of the phage. These insertion mutants are able to assemble particles, but are unable to properly lyse the infected cell. These mutants define a new genetic complementation group which has been designated gene Rz$^-$. In addition to inactivating gene Rz, these TN903 insertions slightly reduce the expression of genes controlling phage assembly. Cells infected with the lambda Rz::Tn903 isolates will, at the normal time of lysis, change their shape from a rod to a sphere.

KO 4 AI 00310-04 D. Storm (University of Washington): Dr. Storm has found that a number of membrane-active antibiotics have served as useful tools for studying the structure and function of biological membranes. EM 49 (recently named octapepin) is a membrane-active peptide antibiotic that has been reported to affect the structure of bacterial membranes. In this study, it is shown that the effects of EM 49 on bacterial metabolism are similar to those of uncouplers of oxidative phosphorylation. EM 49 stimulated bacterial respiration within a narrow concentration range corresponding to minimum inhibitory concentrations and inhibited respiration at concentrations comparable to minimum biocidal concentrations. In addition, the peptide increased membrane proton permeability and lowered the adenosine 5'-triphosphate pool size. Parallel studies done with the related antibiotic polymyxin B demonstrated that the two peptides differed considerably in their effects on bacterial respiration. In contrast to EM 49, polymyxin B did not stimulate respiration at any concentration. It is proposed that the primary action of EM 49 is to disrupt the selective ion permeability of the cytoplasmic membrane, thereby relaxing the membrane potential.

AI 15389-02 F. Tally (New England Medical Center Hospital): The objective of this grant is to study the determinants of virulence in Bacteroides fragilis by: (1) defining transferable drug resistance, (2) developing a genetic system to obtain mutants lacking suspected virulence factors, and (3) developing a phage typing system to investigate the epidemiology of infections caused by B. fragilis.

The past year's studies have provided further insight into the sophisticated systems of genetic exchange found in B. fragilis. These observations suggest an explanation for the widespread development of tetracycline resistance in B. fragilis during the 1960's. Transferable resistance would also provide a mechanism to disseminate clindamycin resistance and high level ampicillin resistance. He has shown that plasmid DNA from B. fragilis can be introduced and maintained in E. coli. This will allow a more rapid analysis of B. fragilis genes encoding for resistance. More importantly, the replication and expression of genetic material from anaerobic bacteria in E. coli demonstrates that there are few barriers for the exchange and expression of DNA in the microbial world. Thus, there may be exchange of genetic material between anaerobic and aerobic bacteria in ecological niches such as the human colon. This raises the possibility that anaerobic bacteria may be a gene pool for resistance determinants. The development of a mutagenic system will allow the study of carbohydrate metabolism in B. fragilis and the development of selective media for the detection of mutants. These studies will lead to the development of methods to study potential virulence factors.

AI 10971-09 M. Higgins (Temple Medical School): Streptococcus mutans is highly susceptible to growth inhibition by benzylpenicillin and other inhibitors of cell wall peptidoglycan synthesis. For example, for the GS-5 strain, increases in culture turbidity are inhibited by as little as 0.02 μg/ml of benzylpenicillin. However, cultures fail to lyse when exposed to up to 250 μg/ml. This tolerance to cellular lysis permitted studies of peptidoglycan, RNA, protein and DNA synthesis and ultrastructure of cells over a range of drug concentrations in the absence of osmotic stabilization. Inhibition of the synthesis of all four of the above polymers was dose dependent. Synthesis of insoluble peptidoglycan was most susceptible; however, substantial but less severe inhibitions of RNA and protein synthesis followed inhibition of peptidoglycan synthesis. Inhibition of DNA synthesis was delayed and minimal. These effects on macromolecular synthesis were compared with quantitative measurements of cellular surface area, volume and shape obtained from computer-assisted reconstructions of surface growth zones and poles observed in electron micrographs. Interestingly, these measurements showed that benzylpenicillin inhibited increases in the surface area and volume of cells that closely paralleled the observed extent of inhibition of peptidoglycan synthesis (rather than that of RNA, protein or DNA synthesis).

AI 09644-11 H. Nikaido (University of California-Berkeley): In further studies, Dr. Nikaido determined that the outer membranes of Gram-negative bacteria is an attractive system for the study of the structure-function relationships in biological membranes. This membrane has the task of regulating the inflow of nutrients and outflow of waste products. Investigations with the aid of mutants showed that there are at least two general pathways for the diffusion of small molecules across the outer membrane: one for hydrophobic and one for hydrophilic compounds. In the case of the "hydrophobic pathway" the hydrophobic compound dissolves in the interior of the membrane and then crosses the membrane in accordance with the partition coefficient. In wild-type forms of enteric bacteria this pathway cannot be used--presumably owing to the absence of regions with phospholipid bilayers. Small hydrophilic molecules, on the other hand, penetrate the membrane through water-filled pores.

In Escherichia coli and Salmonella typhimurium, the cell wall that contains both the outer membrane layer and the peptidoglycan layer acts as a barrier of the molecular sieve type for the penetration of uncharged saccharides. The layers of the cell wall which limited the size of the penetrating molecules were examined by studying the penetration of saccharides into (a) cells whose peptidoglycan layer had been destroyed by lysozyme treatment or growth in the presence of penicillin and (b) isolated outer membrane vesicles. It was found that peptidoglycan-defective cells were similar to intact, plasmolyzed cells in that they allowed a partial penetration of stachyose (molecular weight 666), but essentially excluded saccharides with molecular weights higher than 900 to 1000. It was found also that the isolated outer membrane acted as a penetration barrier for saccharides. These observations led to the conclusion that the outer membrane, rather than peptidoglycan, sets the size limit for the penetration of uncharged, hydrophilic molecules through the E. coli or S. typhimurium cell wall. The isolated outer membrane, however, had an exclusion limit much higher that that found in intact cells. This "leakiness" could be decreased either by the use of mutants producing extremely deficient lipopolysaccharide, or by trypsin treatment of the isolated membrane followed by heating and slow cooling in the presence of Mg^{++}. It is felt that these observations are consistent with the hypothesis that the resealing of the ruptured outer membrane during the isolation procedure is often incomplete, and that cracks and holes thus generated are responsible for the "leakiness" of the isolated membrane vesicles.

(2) Recombinant DNA Molecular Research

During the past few years, the development of certain techniques in the area of molecular biology has made it possible to construct functional DNA molecules *in vitro* which contain segments derived from diverse biological sources. The scientific innovations which led to this technological breakthrough were mostly derivative from basic studies on the mechanism of restriction, which normally acts as a barrier to gene flow among microorganisms, and on the molecular biology and genetics of bacterial plasmids, especially those specifying drug resistance. Grantees of this Institute played a preponderant role in both of these areas. Whereas DNA recombination in nature has depended on random processes, the experimental techniques now available enable the *in vitro* construction and subsequent replication of DNA molecules needed for specific experimental goals. This basic advance, coupled with refinements of the existing technology, should provide greater knowledge of the mechanisms of pathogenicity (at the molecular level) of viral, bacterial, mycotic and parasitic agents. This information, in turn, may lead to improved prevention, diagnosis and treatment of infectious diseases. Notwithstanding the potential benefits of recombinant DNA molecule research, there may be associated potential biohazards which must be avoided by the design, construction and testing of safer host-vector system for use in these studies.

The primary goal of this research is the development and utilization of recombinant DNA molecule technology to increase fundamental knowledge and ultimately enhance control of the etiological agents of infectious diseases. Another goal is the synthesis of a variety of biologically useful substances through the construction of bacterial cells containing functional DNA of either plant or animal origin. An equally important goal is the identification, assessment, and elimination of any and all potential biohazards encountered in the exploitation of this technology.

Research Highlights

AI 08619-13 S. Cohen (Stanford Medical School): The virus that causes hepatitis B, or serum hepatitis, seems to infect only humans in nature, and experimental infection has been achieved in only a few additional mammals. The limited host range of the hepatitis B virus (HBV), and its failure so far to infect tissue culture cells have drastically restricted study of this virus and have hindered development of a vaccine for the serious disease that it causes. Dr Cohen reports the cloning of double-stranded HBV DNA in *Escherichia coli* K12, using the unique *Eco*RI cleavage site on the viral genome to introduce the entire HBV DNA molecule into an *Eco*RI cleavage site within the chloramphenicol (Cm) resistance gene of the pACYC184 plasmid vector.

The ability to clone the total genome of HBV in an *E. coli* K12 strain now allows the amplification and propagation of hepatitis viral DNA species isolated from individual carriers and the production of substantial quantities of HBV DNA for further structural studies. Investigation of endonuclease cleavage patterns of cloned Dane particle DNA derived from different carriers showing the same viral subtype, should elucidate the extent of heterogeneity of the HBV genome in such carriers and should enable correlation of DNA primary structure with subtype specificity.

In addition, the prospect of expression in bacterial cells of peptides encoded by the cloned HBV viral genome potentially provides a means of synthesizing large amounts of viral surface antigen suitable for use in production of a vaccine for viral hepatitis.

Dr. Cohen has also studied replication control and plasmid incompatibility. Because of their small size, stable autonomous maintenance and dispensable nature, bacterial plasmids offer an attractive model system for studying the mechanism by which bacterial DNA synthesis is regulated.

AI 10311-11 D. Dubnau (Public Health Research Institute of New York): The minicell system of *Bacillus subtilis* has been used to study the expression of plasmid genes using several R plasmids derived from *Staphylococcus aureus*. pE194, pC194, and pUB110 as well as several mutant and *in vitro* recombinant derivatives of these plasmids segregate into minicells. A copy control mutant of pE194 was used to show that the extent of segregation is proportional to the copy number. The polypeptides specified by these plasmids were examined by SDS-polyacrylamide gel electrophoresis. Six proteins specified by pE194, an erythromycin resistance plasmid, were identified using *cop* mutants. These comprise about 90% of the potential coding capacity of the 2.4-Mdal pE194 plasmid. One of these proteins (29,000 daltons) is inducible by erythromycin in the wild type pE194 but is synthesized constitutively in a mutant derivative which also expresses antibiotic resistance constitutively. Several other proteins are detected only in copy control mutants. pUB110, a kanamycin resistance plasmid, expresses three major proteins which comprise 50% of the coding capacity of this 3.0-Mdal plasmid. Two additional minor proteins are occasionally observed. pC194 (2.0 Mdal), which confers chloramphenicol resistance, expresses two polypeptides comprising about 25% of its coding capacity. One of these polypeptides (22,000 daltons) is inducible by chloramphenicol. pBD9, an *in vitro* composite of pUB110 and pE194, probably expresses all of the major parental plasmid proteins with the exception of one from pUB110 and one from pE194.

AI 07194-15 D. Helinski (University of California-San Diego): To construct plasmid cloning vehicles for gram-negative bacteria distantly related to *E. coli*, particular advantage was taken of the inc P-1 plasmid RK2. This plasmid can be transferred and stably maintained in a variety of gram-negative bacteria, including the medically important genera *Haemophilus*, *Neisseria*, *Vibrio* and *Yersinia*. The three regions of the naturally occurring RK2 plasmid which are essential for replication have been incorporated into the plasmid cloning vehicle designated pRK290. This plasmid is a 20 kilobase tetracycline-resistant deletion derivative of RK2. It is mobilizable but not conjugative, and has two unique restriction enzyme sites, *BglII* and *EcoRI*. Both of these sites have been used successfully for cloning large DNA inserts. pRK290 can be complemented for conjugal transfer by the helper plasmid pRK2013. This latter plasmid is a kanamycin-resistant hybrid containing ColE1 linked to a segment of RK2 DNA that specifies transmissibility properties of this plasmid. pRK2013 is self-transmissible in E. coli, but cannot be stably maintained in other gram negative hosts due to the narrow host range of the ColE1 replicon. High frequency transfer (1-10% per recipient) of foreign DNA inserted into pRK290 can be achieved from E. coli to other distantly related gram negative bacteria with this binary vehicle system. The non-self-transmissible nature of the vector itself provides a high level of biological containment.

AI 17211-01 J. Ferretti (University of Oklahoma Health Sciences Center): A physical map of the streptococcal macrolide, lincomycin and streptogramin B resistance plasmid pDB101 was constructed using six different restriction endonucleases. Ten recognition sites were found for Hind III, seven for Hind II, eight for Hae II, and one each for Eco RI, Hpa II, and Kpn I. The localization of the restriction clevage sites was determined by double and triple digestions of the plasmid DNA or sequential digestions of partial cleavage products and isolated restriction fragments, and all sites were aligned with a single Eco RI reference site. Plasmid pDB101 meets all requirements essential for a potential molecular cloning vehicle in streptococci; i.e., single restriction sites, an MLS-selection marker, and a multiple plasmid copy number. The vector plasmid described makes it possible to clone selectively any fragment of DNA cleaved with Eco RI, Hpa II, or Kpn I, or, since the sites are close to each other in map position, any combination of two of these restriction enzymes.

Parasitology

The 14th annual meeting of the U.S.-Japan Parasitic Diseases Panel was held in New Orleans, Louisiana on August 13-15, 1979. Approximately 50 participants, 10 of whom came from Japan, heard 37 papers covering various aspects of studies on schistosomiasis and filariasis. Four mini-symposia were organized: "Schistosome antigens," Schistosome chemotherapy," "Filariasis in Latin America" and "Immediate hypersensitivity in filarial and schistosome infections." The final day of the meeting included a visit to the Delta Regional Primate Research Center of Tulane University in Covington, Louisiana. In addition to the formal scientific sessions, many of the U.S. participants took advantage of opportunities to develop potential collaborative research relationships with their counterparts.

The Parasitology Section was also represented at the November 1979 meeting of the American Society of Tropical Medicine and Hygiene, each of the three meetings of the Tropical Medicine and Parasitology Study Section, and the April 1980 workshop on "Application of Recombinant DNA Technology to Protozoology."

In February 1980, one Tropical Disease Research Unit (TDRU) program project grant was funded. The basic objective of this new initiative is to bring together relevant biomedical knowledge and technology in a multi-disciplinary attack on the world's tropical and parasitic diseases. This TDRU at Harvard University will involve an immunological approach to the study of leishmaniasis, South American trypanosomiasis, filariasis, and schistosomiasis. The major objectives will be to provide diagnostic tools, to contribute to a better understanding of humoral and cell-mediated mechanisms, and to provide means of defining protective antigens and adjuvants necessary to develop safe and effective vaccines.

Program Summary

In addition to the basic free-ranging research of relevance to the Institute, there are two structured programs supported by this section of the Branch - biological regulation of vectors and immunology of parasitic infections.

(1) Biological Regulation of Vectors

This program has as its goal the advancement of fundamental studies which might lead to effective methods of biological regulation of vectors. For the past 30 years, control of pests and disease vectors has been based primarily on the use of synthetic organic compounds which had the "advantages" of long residual action and toxicity to a broad spectrum of target organisms. It has now been shown that because of these very characteristics, many of these pesticides are more deleterious than beneficial, when all effects on man and his environment are considered. Furthermore, resistance to broad spectrum chemical pesticides has reduced their effectiveness in many vector control programs. For these reasons the search for alternative methods of pest control has become imperative, and it is generally agreed that the best approaches will consist of integrated pest management programs which combine biological control, in the broadest sense, with the judicious use of more specific chemicals and management of the physical environment. This approach to vector control must be based on adequate information about the ecology of the target organism, the environment in which the control program is to be conducted, effects of control measures on non-target organisms in the environment, and the biology of the disease organisms being transmitted.

Research Highlights

AI 15605-02 D. Molloy (State University of New York, Albany): Current data demonstrate that the nematode Neoaplectana carpocapsae shows considerable promise as a biological control agent of black flies. The nematode is highly virulent to Simulium (the vector of onchocerciasis), causing rapid death and high mortalities. Concern over non-target susceptibility appears to be largely unjustified. Moreover, the likelihood of stream establishment seems remote given the nematode's limited reproductive capacity in aquatic invertebrates.

AI 02753-21 G. Craig (Notre Dame University): The large non-vector mosquito Toxorhynchites rutilis was released in local woodlots in Indiana for the control of the vector mosquito, Aedes triseriatus. Seventy female Toxorhynchites were released in a woodlot of 10 acres with 136 treeholes. The Toxorhynchites laid eggs in every treehole and two additional generations of T. rutilis were produced in a three month period. The Aedes population was reduced by half. Another accomplishment in biocontrol potential comes from a field release experiment with Aedes triseriatus infected with a new strain of the gregarine protozoan, Ascocystis barretti. Using a dye-marker release method it was shown that after 10 days, the infected mosquito had virtually disappeared whereas the uninfected mosquitoes were abundant.

AI 10187-10 K. Nakanishi (Columbia University): Extract of the bark of an East African tree, the Warburgia, is being tested as an insect anti-feedant which irreversibly turns off an insect's sense of taste when it nibbles on a treated plant. Not only are antifeedants nontoxic to higher forms of life, but, unlike DDT and many other synthetic pesticides, they break down readily in the environment. In addition, it has been shown that this antifeedant kills the snail vector of schistosomiasis.

AI 14306-03 E. Davidson (Arizona State University): Several insecticidal fractions have been isolated from Bacillus sphaericus cells. The insecticidal activity seems to be in the cell membrane-cell wall fraction and is released during digestion of the bacterial cell during digestion by the mosquito larva. The toxin is probably a single protein with a molecular weight less than 100,000 daltons. Current studies include the effects of the toxin on insect cell and organ cultures and the investigation of activation, subunit composition, and cellular binding. B. sphaericus has been shown to have insecticidal action against the malaria vector, Anopheles, the yellow fever and dengue vector Aedes aegypti and various Culex species which transmit encephalitis and filariasis.

(2) Immunology of Parasitic Infections

The complexity of structure and function of parasites has made the study of the immunology of these infectious agents exceptionally challenging and rewarding. Exciting opportunities for the elucidation of mechanisms and manifestations of immunological responses to parasites now exist as the result of the impressive developments in immunology in recent years. Major ultimate goals of studies on the immunology of parasitic infections are the development of effective vaccines for the prevention of parasitic diseases (such as malaria, schistosomiasis, and filariasis), the intervention in the host reponse to prevent or ameliorate disease processes which are immunologically mediated, and the development or improvement of immunodiagnostic procedures for parasitic infections, especially as they relate to the immune status of the host.

A related goal of these studies is to contribute to an understanding and solution of basic and clinical problems associated with other disease entities, especially immunological disorders and hypersensitivity states. A number of parasitic infections are excellent models for such studies as (a) the mechanisms of intracellular immunity, (b) the enhancement or suppression of concurrent infections or tumor development, (c) immunopathological mechanisms, (d) development of disease processes in immunosuppressed or immunostimulated hosts, (e) the biochemical and genetic mechanisms for the development of pathogen variants with different immunological characteristics, (f) the genetic basis for variations in host reponse, and (g) the role of IgE and other cytotropic antibodies in hypersensitivity.

AI 13366-04 R. Nussenzweig (New York University): The application of monoclonal antibody procedures has facilitated studies of the protective antigen of Plasmodium berghei sporozoites. Incubation of intact sporozoites with monoclonal antisporozoite antibody results in the formation of a surface coat surrounding the entire parasite. Sporozoite infectivity was also abolished by in vitro incubation of sporozoites with monospecific antibody. These studies are being extended in an effort to characterize, isolate, and purify the protective antigens of the human parasite - Plasmodium falciparum, so that these antigens might eventually be used in a malaria vaccine.

AI 02631-22 F. von Lichtenberg (Harvard): In a study of the immunological control of schistosome granuloma formation, serum gamma globulin shows the double effects of protecting recipient liver cells from microvesicular fatty degeneration and of restoring eosinophilic granulocytes to their liver granulomas. However, gamma globulin was not absolutely effective in this regard as was whole chronic infections serum.

AI 14490-02 D. McGregor (Cornell University): In a study of "rapid expulsion" as an expression of host immunity to *Trichinella spiralis*, it was shown that either enteral or parenteral stages of the parasite are capable of inducing this expulsion of a larval challenge infection. Genetic studies have indicated that the capacity to express rapid expulsion is inherited as a dominant characteristic.

AI 13767-03 D. Weiner (University of Pennsylvania): In a study of the relationship of the antifilarial action of diethylcarbamazine and the immune system, evidence has indicated that the immune system may be involved in two ways. First, the diethylcarbamazine may strip a protective coat of immune origin from the microfilariae, then the immune elimination of microfilariae may take place.

AI 14848-02 F. Kierszenbaum (Michigan State University): Congenitally athymic mice were shown to be significantly more susceptible to *Trypanosoma cruzi* infection than their thymus-bearing litter mates, as measured by increased parasitemia, mortality rate, and by shortened survival time. In addition, transplantation of neonatal thymus into athymic mice reestablished normal levels of resistance to *T. cruzi*. These results indicate that host defense mechanisms active in experimental Chagas' disease are under thymic control.

(3) General Parasitology Research Highlights

AI 15663-02 J. Marr (St. Louis University): Enzymological similarities were demonstrated between the pathogenic leishmania, *T. cruzi* and the African trypanosomes with respect to their metabolism of pyrazolopyrimidines (such as allopurinal). This hitherto unsuspected relationship, if exploited with further study of purine chemistry, may establish some common pathways useful in chemotherapy. In addition it was shown that allopurinal ribonucleoside (HPPR) is much more active that the parent compound (HPP) against *Leishmania*. This finding opens the possibility of using pyrazolopyrimidine nucleosides as chemotherapeutic agents since they are more stable and are nontoxic, unlike purine nucleosides.

AI 10588-09 F. Anderson (Brigham Young University): As part of a study of the epidemiology of echinococcosis, a coloring book (for grades 1-4) on hydatid disease has been developed. The last 2 pages of the coloring book contain a 10 question quiz. Over 700 students in 21 classes took the quiz both before and after reading the coloring book. Twice as many questions were answered correctly by these students in the post-coloring book exam than in the pre-coloring book test. Strong correlations between the level of knowledge of hydatid disease among the community and some of the preventive measures taken by sheepmen have led the investigators to be optimistic about eventual control.

AI 15864-02 M. Dresden (Baylor University): An acid protease of adult *Schistosoma mansoni* has been purified and characterized with respect to optimal pH, molecular weight, requirements for thiol reagents and the effect of temperature. This study is significant from two standpoints: (1) a knowledge of the function of this enzyme should allow the testing of specific inhibitors to interfere with the parasite's metabolism and (2) the enzyme can be used in the diagnosis of schistosomiasis. The enzyme makes up a substantial portion of the protein of the parasite and its presence should reflect the degree of infection.

AI 11942-06 M. Muller (Rockefeller University): An aerobic *in vitro* test has been devised to detect metronidazole resistance in *Trichomonas vaginalis*. The availability of this test will permit studies to assess the contribution of drug resistance to the known 10% failure rate of metronidazole treatment in human trichomoniasis. Other findings underscore more and more the metabolic differences between trichomonads and their mammalian hosts and will form a basis for rational development of new chemotherapeutic agents.

Contract Activity

The schistosomiasis supply contract was readvertised and a new 5 year award made to the University of Lowell. This service has continued to be utilized extensively by most schistosomiasis researchers in this country. The contract provides all three human schistosome species and their vector snails.

The filariasis supply contract was also readvertised this year and a 5 year award made to the University of Georgia. This contract provides research workers with five filarial genera and their vectors. Acceleration of research on filariasis is in large part directly attributable to this service.

A new contract for the development of techniques for the radio-labelling of schistosome and filarial larvae was awarded to Cornell University in mid 1979. A wide variety of gamma-emitting isotopes have been tested and labelling techniques are being refined. The interaction and collaboration of a parasitologist, and entomologist, a radiobiologist, an immunochemist, and an immunoparasitologist is proving to be quite productive in the attempt to solve a very difficult and yet significant problem in parasitic diseases.

EXTRAMURAL ACTIVITIES PROGRAM

NIAID

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REPORT OF THE DIRECTOR, EAP

A. Introduction

The FY 1980 workload of the Extramural Activities Program of the NIAID was similar in most work counts to that of fiscal year 1979. However the decade of the 80s, which opened with the much heralded reduction in federal paperwork saw no impact of this reduction on EAP activities because of increased reporting requirements as a result of Departmental and other regulations, the need for closer cost accounting, and the requirements for stricter adherence to deadlines. As in the past, this was handled with the same number of staff members.

Although there was an increased budget there was also an increased number of applications, therefore there was no increase in the rate of payment of regular research projects, fellowships and training grants. It was also disappointing to note that it may be the last year for some time that we are able to award competing fellowships. The training report section of this Annual Report will provide the data showing the rapid decline in our training program as forecast by present administration budget plans. Even in fiscal year 1980 the increased cost for stipends and institutional support in the training programs seriously curtailed the number of fellowships and training grants which could be awarded.

In the regular research program the NIAID funded research grant applications to a normalized score of 215. In addition, a system was initiated to enable the Institute to pay additional meritorious research projects of special interest to the NIAID. Under this plan, 36 applications were identified for selective funding. In all, approximately 440 competing research projects were awarded in FY 1980 with an estimated pay rate of 37 percent.

B. The Review of Research Proposals

The Program and Project Review Branch was able to use word processing equipment and additional help from the Office of the Director, EAP, to complete its assignments on time this year. As in the past, all their reviews were sustained at the next level of review. This year it seemed quite clear that the separation of the review process from program was working as it was originally designed in the reorganization of the Institute.

C. Personnel

There were four departures from the Extramural Activities Program and nine new appointments during FY 1979. The lag time for the processing of appointments of new employees remains at least the same as it was last year and perhaps even greater. The loss of two to three man years in the processing of these appointments continues to hamper the accomplishment of the EAP objectives.

Employees who have left EAP

Lalita Anthony
Christiane Karis
Francis Hisaoka
Rosemary Spicer

New Appointments

Darlene Michalski
Mary Lou Davis
Dianne Davis
Catherine Roberts
Dorothy Leggin
Peter Mullin
Delores Gibson
Judy Vann
Diane Kaufman

Awards

Celeste Mayer
Mabel Battistone
Rosemary Spicer
Etta Kidwell
Fran Schlademan
Mattie Tynan
Susan Bozak
Sylvia Cunningham
Dolly Knadler

GMB Group Award

D. Manpower Development

Two hundred and fifteen (215) Individual Fellowship (F32) applications were reviewed during the fiscal year (1980). Of these 215 applications 192 or 89% were approved (see Table I).

TABLE I

Individual (F32) Fellowship Applications Reviewed FY 1980

Total No. Applications	Applicants by Degree		Approved Applicants	
	M.D.	Ph.D	M.D.	Ph.D.
215* 100%	42 (20%)	173 (80%)	38 (17%)	154** (72%)

*3 F33's included.
(F33=Senior Fellowship)

**1 F33 included.

It is estimated that only 36 of the successful applicants will be awarded by the close of the fiscal year (priority score cut-off 141). Included in these applications were three F33 or Senior Fellowship proposals of which only one was approved which had a priority score in the payable range.

Table II reflects the distribution of the reviewed training grants among the two major programs.

TABLE II

Training Grants (T32) Reviewed FY 1980

Total No. Applications	Applicants by Program		Total No. Trainees Recommended			
	IAIDP	MIDP	IAIDP		MIDP	
			Pre	Post	Pre	Post
22	11	11	11	11	11	11

On June 30, 1980 four training grants were terminated leaving 76 grants. It is planned that three new training grants will be awarded for fiscal year 1980 thus maintaining a portfolio of 79 training grants during fiscal year 1981 which includes one short term (F35) training grant. However, because of the President's zero budget for training in fiscal year 1981 the NIAID will terminate 22 training grants leaving a portfolio of 57 training grants in fiscal year 1982. Should the budgetary constraints continue into fiscal year 1982 the number of training grants and individual fellowships will be reduced drastically. The shortfall of the individual fellowships will begin to manifest itself significantly in fiscal years 1981 and 1982 if denial of support continues. Table III shows the estimated total number of trainees, fellowships and training grants that will be supported during the fiscal years 1980-1982 should the President's budget have zero dollars for training.

TABLE III

Estimated Fellowships and Training FTE's

FY 1980, '81, '82

	1980	1981	1982
Postdoctoral:			
F32	121	77	44
T32	263	171	102
Subtotal:	384	248	146
Predoctoral:			
T32 only	131	122	81
TOTAL FTE's:	515	370	227
PLUS: T35 (Short term Trainees)	24	28	32
Training Grants:			
T32	78	56	33
T35	1	1	1
	79	57	34

E. Manpower Survey Conducted by the American Society for Microbiology

The survey was completed and a draft report submitted. There were numerous changes suggested to the American Society for Microbiology. These were incorporated in the final report which is now being examined for acceptance. Highlights of the analysis of the data show that the American Society for Microbiology membership is fully employed; minority grants comprise about one tenth of the respondents (total respondents analyzed 15,665). Microbiologists holding the B.S. degree make up the largest population in the industrial, clinical, and state government sectors, while Ph.D. microbiologists comprise the largest segment in academia, and the federal government. Approximately 34% of all respondents work in basic and applied research and 27% of the respondents receive federal support for their work, Ph.D.s receiving the highest level of federal support.

F. Committee Management

The Committee Management Officer who was appointed at the beginning of FY 1980 labored for approximately nine months without the assistance which had formerly been provided Committee Management Officers. As we approach the last quarter of the fiscal year, having completed three Councils, it has been possible to obtain a temporary clerical person for this office. We are very pleased with the way in which the new Committee Management Officer has covered these two positions with assistance from the secretary of the Training Officer and others. It is hoped that this person will continue uninterrupted in this position because the temporary measures for filing travel claims and arranging reimbursement for consultants and Council members cannot be allowed to continue with the unfortunate delays that have been tolerated this fiscal year. Council and committee appointments have been made on schedule and the support this office has supplied for the advisory activities for the Institute has been very successful.

G. Conference Proposals

The EAP review of unsolicited contract proposals from the Fogarty International Center (FIC) and from the Division of Research Grants has continued at about the same level as in fiscal year 1979. During this year a larger number of applications from the FIC were returned to them without review because of lack of Institute relevance or other policy problems. Table IV shows the prospecti reviewed be NIAID in FY 1980.

The Director of the Extramural Activities Program was a member of a committee to examine the activities of the Conference and Seminar Program Branch of the FIC. The Task Force which reviewed the overall mission of the FIC has recommended the removal of conference management for the NIH as a FIC activity. However the committee suggested that the role of broker played by this office is extremely important to NIH management and review of conferences and an important service to the scientific community. They recommended that if this service is not retained in FIC that it find some other focus at the NIH.

TABLE IV

CONFERENCE/WORKSHOP PROSPECTI REVIEWED BY NIAID
FY '80

Received			113 1/
From Fogarty International Center (FIC)		109	
Sponsorship & Funds	108		
Sponsorship Only	1		
Directly by Institute		1	
From DRG (R-13's)		3	
Not Reviewed			34
Returned to FIC due to lack of Institute relevance		28	
Returned to FIC due to Institute policy		3	
FIC Action--Withdrawal/Disapproval/Deferral		3	
Reviewed			67
Approved		49	
Sponsorship & Funds	41		
Sponsorship Only	1		
Approved (No Funds)	7		
Disapproved		18	
Sponsorship & Funds	18		
Sponsorship Only	0		
To Be Reviewed			10

FUNDED OR SCHEDULED FOR FY '80 FUNDING (Thru July 80)

	IAIDP		MIDP		Other NIAID		TOTAL	
	#	$	#	$	#	$	#	$
R-13 Grant Applications	0	0	2	$ 29,105	0	0	2	$ 29,105
Other Extramural Prospecti	6	$58,000	23	$143,022	10	$47,500	39	$248,522
MAXIMUM TOTAL	6	$58,000	25	$172,127	10	$47,500	41	$277,627

1/ Includes some which were received in FY '79 but reviewed in FY '80

Prepared by:
Mildred S. Warfield 7/22/80 (Revised 8/12/80)

I. CONTRACT MANAGEMENT BRANCH

The Contract Management Branch provides management services to the Institute's Research Program including solicitation, negotiation, award, and administration of all Institute research contracts.

The CMB will continue to implement contract policies and procedures promulgated by higher procurement authority. The CMB works very closely with the Program and Project Review Branch and the various NIAID Project Officers and provides contract management expertise necessary in order to continue to have an effective contract program.

Through effective contract management, NIAID has continued to increase its percentage of competitive awards from 26.2% in FY '78 to 37.3% in FY '79 with an estimate of 50% for FY '80. NIAID is one of the few Institutes which exceeded the competitive goals established by Division of Contracts and Grants.

Other accomplishments for FY '80 are:

1. Even distribution of contract awards within the fiscal year.
2. Made contract awards in all three socio-economic programs - 8(a), Small Business Administration and Labor Surplus set-Aside.
3. Led all Institutes in the total percentage in meeting its goals established by Division of Contracts and Grants in closing-out of contracts.

In FY '80 CMB received approval for 21 contract requests and issued 862 requests for proposals to organizations which expressed interest. In response to the above advertisements, 58 proposals were received and 22 new competitive contract awards were made. It is estimated that there will be 160 active contracts with an FY '80 allocation of $14,500,000.

II. GRANTS MANAGEMENT BRANCH

Fiscal Year 1980 was a year marked by change and increased workload for the GMB. The grant programs at NIH represent approximately 2/3rds of the total budget and exceeds $2 billion annually. In the NIAID, Grants also represent approximately 2/3rds of the Institute's total budget, with over 1,800 grants for approximately $140 million awarded in FY 80. The GMB is responsible for the fiscal and administrative management of all grants and awards issued by the NIAID. The GMB works very closely with the programmatic divisions and provides the fiscal and administrative expertise necessary for effective program management. The GMB also serves as an interpreter of grant policy and procedure issued by the several echelons within the DHHS.

To report, briefly, on changes cited in the 1979 report, we are pleased to say that: 1) the loss of the categorical Report of Expenditures, which was to have been replaced by the non-categorical Financial Status Report, did not materialize to the extent anticipated and, in fact, to a large degree we continue to receive the categorical ROE's; and 2) that "savings" resulting from the new project-period concept which enables the awarding component to use the total unexpended balance from one project-period segment as a funding offset to another, will exceed $250,000 for FY '80.

One change in FY '79 that impacted or may impact on GMB in FY '80 was stipend and allowance increases for NRSA trainees and fellows. The effective date of the increases was July 1, 1980; however, since most trainee and fellow periods of support were for periods with start-dates other than July 1, there existed the need for many additional administrative adjustments, such as revised Statement of Appointment forms, Activation Notices, APAC's, and other requirements dictated by the NRSA. This generated considerable additional work while at the same time slowing down the process of making NRSA awards.

The staff of GMB continues to make significant contributions as members and chairpersons of policy and procedure work groups, committees and subcommittees at the NIAID and at the NIH. Examples of such activity include: NIH Grants Management Self Appraisal and Training; PHS Grants Policy Statement; NIH Committee on Short-Term Training; NIH Working Groups on questions of "Automatic" Carryover, Intermingling of Funds Between Grants, and Changes in Requirements for Expenditure Reporting. Additionally The Chief, GMB, participated as a faculty member at seminars where grantee personnel were in attendance, such as the Seminar on Administration of NIH Grant Programs, at the University of Maryland, Baltimore, which was held for the northeast region of the U.S. The Chief, GMB, was also a faculty member for numerous PHS and NIH training courses attended by PHS and NIH personnel. Further, the Chief, GMB, continues to serve as a member of the GMAC, and was recently appointed to a three-year term as a member of the NIH STEP Committee.

In summary, the Branch performed extremely well in FY '80. In keeping with the projected Branch philosophy employees were encouraged to broaden their horizons beyond their day-to-day routine. Classes and seminars were attended with more regularity. Special emphasis was placed on furthering the partnership role between the grants management specialists and our program administrator counterparts. More project site visits were attended. In general the staff of GMB took a significant step forward in their individual development while maintaining a high level of excellence in pursuit of the day to day duties and responsibilities that encompass all grant programs funded by the NIAID.

III. PROGRAM ANALYSIS AND EVALUATION BRANCH

The Branch is responsible for the NIAID scientific and administrative management information system used to develop reports and support the Office of the Director, NIAID Program Directors, and the NIAID staff data and information requests. The Branch also serves as a focal point for management and budgetary data for Extramural programs; referral liaison with the Division of Research Grants; NIAID ADP Security Compliance Coordinator; ADP Liaison with other Institutes and NIH; and reporting of non-program specific reports such as Prevention and Toxicology or Institute-wide reports for National Science Foundation or NIH.

A new management data base is being developed by Branch personnel augmented by one-person year, non-tenured professional support. All competing grants applications (663), non-competing grants (1026), contracts (134), and Intramural projects (111) have been entered into the new multi-axis coding system, MACS.

Percentage in Clinical Trials, Research Objectives, Special Research Portfolios and Basic, Applied or Developmental Research Areas are now included in the NIAID system for each grant, contract, or Intramural project.

In order to produce more timely and accurate reports and enhance data credibility and consistency, a procedure for the control and documentation of the data base has been developed by PAEB. Target date for implementation by Program and PAEB staff is fiscal year 1981. Health Science Administrators will be requested to review the MACS codes assigned by the PAS staff to projects in their Programs, and then to submit any modifications to PAEB.

PAEB coordinated Institute's efforts to provide a baseline profile on ADP Security Policy Compliance. The NIAID portion of the HHS ADP Systems Security Policy Compliance Report was prepared in July. The baseline profile covering 20 site compliance reports for an inventory of 44 terminals or ADP hardware will be updated quarterly. The ADP Systems Security Coordinator (SSC) is working with OPM-NIH to develop standards, waivers, training and security awareness programs to implement ADP Systems Security Policies as directed by the Office of Management and Budget. The SSC serves as member of a task force to evaluate the PHS ADP Systems Security Review Program.

A. Fiscal Management and Analysis Section

In compliance with the policies of the DHHS, this office enlisted the help of the EAP in an effort to reduce the heavy workload and large expenditure of grant funds in the fourth quarter of the fiscal year. In spite of the willing cooperation of all offices involved, the number of grants awarded and dollars obligated continue to be inordinately heavy in the last quarter due to the timing of the largest Council (late May) and the large number of noncompeting commitments with start dates in August and September.

B. Data Control Section

The functions of the Data Control Section, using the NIH and Parklawn computer facilities, include generating management, budgetary, and programmatic reports for the Extramural Activities Program, the Immunology, Allergic and Immunologic Diseases Program, and the Microbiology and Infectious Diseases Program, as well as other areas of NIAID such as the Information Office, the Budget Office, and the Office of the Director in the areas of grants, contracts and Intramural activities.

The 1979 Awards and Projects Activities Book was prepared using the Multi-Axis Code System, a new management information data system. All areas of NIAID support were identified by the new scientific identification, including, for the first time, the Intramural projects.

ADP Security is being reviewed, and, to the extent determined necessary, will be implemented into Data Control Section procedures.

C. Program Analysis Section

During the past year, the Program Analysis Section (PAS) has combined the utilization of the Multi-Axis Coding System (MACS) with the validation and standardization of the data within the system. In fiscal year 1980 PAS classified the scientific and programmatic content of approximately 680 competing applications through a normalized score of 250. This information was subsequently incorporated by the Data Control Section with existing data to produce a data base of approximately 1800 active grants for FY 1980.

PAS staff is continuing to sharpen Program descriptions of content, to develop selection criteria, and to standardize request formats so that NIAID's data collection system for handling scientific and programmatic management information is both flexible and responsive.

An increase of interest in the Trans-NIH Health Research Problems and Similar Portfolios by the Administration, members of Congress, and the general public has placed upon PAEB the responsibility of insuring complete and comparable data when reporting these activities: Arthritis; Blood Related Diseases; Bronchopulmonary Disorders; Cerebral Palsy; Cystic Fibrosis; Diabetes; Digestive Diseases; Dermatology; Epidemiology; Genetic Diseases; Hepatitis; International Health Research; Kidney Diseases; Lupus; Nutrition; Prevention; Recombinant DNA: Reye's Syndrome; SIDS; Toxicology. Additional analytical reports prepared by PAEB staff covered NIAID research in STD, Immunology and Virology.

IV. PROGRAM AND PROJECT REVIEW BRANCH

A. Introduction

FY 1980 continued to be an active year for the Program and Project Review Branch, with the Microbiology and Infectious Diseases Advisory Committee (MIDAC) and the Allergy and Clinical Immunology Research Committee (AIRC) having full review workloads (the workload is shown in Table V). The Transplantation Biology and Immunology Committee (TBIC) though having a light review load this year, played an active advisory role in the KTHS Program and the serum bank, and cleared the concept of the establishment of Transplantation Immunology Centers (which also received full endorsement from the National Advisory Allergy and Infectious Diseases Council). For the first time, the PPRB conducted a formal orientation of new committee members, which was successful and was appreciated by the new members. We plan to do this annually in concert with the June Committee meetings. Committee members and other consultants were made particularly cognizant of the conflict of interest statutes and their serious implications. Unswerving compliance with the statutes regarding conflict of interest may have a disturbing consequence on peer review because there might be projects or programs where all available experts may be considered in conflict, e.g., the influenza program.

Committee members have been urged to submit names of qualified individuals, particularly women and minority members, to the NIH Consultant File. This is a computerized repository containing currently up to 17,000 names of biomedical scientists who responded to the NIH invitation for names for consideration for advisory group membership. Executive secretaries have been encouraged to use this File.

The guidelines for reviewers for the review of contract proposals have been revised as of July 1980. The Program Project Brochure is undergoing revision (to make it agree with the revised Form 398).

The Branch continued to be plagued with inadequate support personnel. The grants clerk serving the AIRC and TBIC left for another job and the position has been vacant since February, resulting in a number of minor logistic problems in the conduct of site visits on applications reviewed by AIRC at its March meeting. Immediately prior to the January 1980 Council the number of staff in the Review Services Unit (RSU) was down to two. The number has gradually risen to five, with new recruits who have had to be trained. Because of the heavy workloads of the MIDAC and the AIRC for their March meetings, an RSU employee was reassigned to assist the executive secretaries, in addition to her grant processing responsibilities.

Problems encountered last year in the coordination of clinical trials were not evident this year, and we met the DRG deadline for submission of data.

TABLE V

REVIEWS BY MIDAC, AIRC, AND TBIC
FY 1980

	NUMBER OF APPLICATIONS/PROPOSALS REVIEWED		
	MIDAC	AIRC	TBIC
CONTRACTS			
Solicited (RFP Responses and Renewals):			
Full Committee	35 (3)	0	3 (1)
Ad Hoc	31 (13)	0	11 (4)
Unsolicited	1*	0	1**
Total Proposals Reviewed	66	0	15
GRANTS			
P01/P50			
Solicited (RFA Responses)	11	12	0
Unsolicited	1	0	0
Supplements	1	0	0
T32	9	6	4
K07	-	3	-
PROJECT SITE VISITS (P50/P01)	3	12	0
PROGRAM EVALUATION	1	0	0
CONCEPT CLEARANCE (FULL COMMITTEE)	0	0	1

( ) = Number of meetings

* = Ad hoc meeting

** = Reviewed by full TBIC

B. Microbiology and Infectious Diseases Advisory Committee

Contrary to hopes for some reprieve, the MIDAC continued to have heavy review workloads for the meetings held between October 1979 through September 1980. The Committee formally held three meetings (the October meeting was held in two sections, in October and in December, respectively, due to the heavy workload for that round).

Grants: The MIDAC reviewed 12 program project applications (11 were in response to two RFAs) plus one supplement to a program project, and nine institutional fellowship applications, approving seven program projects, one supplemental application, and seven institutional fellowship applications. The total dollars reviewed for grants were $5,821,919 and the total dollars approved were $2,601,802.

Contracts: The full MIDAC reviewed 35 contract proposals involving 12 RFPs, resulting in 16 proposals being awarded. The total dollars competitively reviewed by MIDAC for contracts was $7,978,348 and the total amount awarded was $4,488,652. Thirteen ad hoc meetings were conducted to review a total of 31 contract proposals. Of these ad hoc meetings, eight were conducted by Dr. Fisher for a total of 19 proposals reviewed and four were conducted by Dr. Bourgeois. Of note is that three of the 13 ad hoc reviews were for new, competing proposals. Only one unsolicited proposal was reviewed this fiscal year for the MIDP.

Program Evaluation: The Otitis Media Vaccine program was reviewed and evaluated by the MIDAC at its March 1980 meeting. (The Enteric Diseases Program will undergo MIDAC evaluation at its October meeting.)

Concept Clearance: No concepts were formally cleared by the full Committee. Two Committee members reported on a NIAID-sponsored Workshop they attended on Influenza B infections.

C. Allergy and Clinical Immunology Research Committee

The AIRC met three times during the period of November 1979 through September 1980, reviewing 12 program project and center, six institutional fellowship and three Allergic Diseases Academic Award applications, of which ten, six, and two, respectively, were approved. The total dollars reviewed were $2,785,025 and the total dollars approved were $1,574,998. No contract proposals were reviewed. The program projects and applications reviewed were in the areas of Asthma and Allergic Diseases, Mechanisms of Immunologic Diseases, and Immunodermatologic Diseases. These were all site visited. Project site visits have been conducted or are underway on the five program project applications that will be reviewed by the AIRC at its October 1980 meeting.

No contract proposals were reviewed by the full committee nor by ad hoc groups during this fiscal year. An RFP initially contemplated on Diagnostic Materials for Food Allergies was cancelled.

> Program Advice: Program staff sought the Committee's advice on a variety of issues including C_3 standards, fungal allergens, the K07 Program, antisera to IgG subclasses and the immunodermatology program.

D. Transplantation Biology and Immunology Committee

The Transplantation Biology and Immunology Committee met twice in FY 1980, reviewing four institutional fellowship applications and approving three. The total dollars reviewed for institutional fellowships was $480,605 and the dollars approved was $294,974. The TIC also reviewed at its March 1980 meeting four contract proposals (two RFPs and one unsolicited proposal), for a total requested amount of $832,385. The unsolicited proposal and one solicited proposal (Hybridoma Cell Lines) were awarded for a total amount of $345,803. In addition, Dr. Sheffield conducted four ad hoc meetings reviewing a total of 11 contract proposals, two of which were for the intramural program and two for the Transplantation Biology Extramural Program.

> Program Advice: Program staff sought advice from the TBIC on a variety of issues including the KTHS program, the serum bank, HLA tissue typing trays and the approval of selected workshops.
>
> Concept Clearance: The Committee cleared the concept of NIAID-sponsored Transplantation Immunology Centers.

E. Review Services Unit

The total number of grant applications processed by the RSU and reviewed by NAAID Council during FY 1980 was 1,646. An additional 142 applications for short term training were processed. Fellowship applications processed by the RSU and reviewed by the NIAID Fellowship Review numbered 230. Just before the January Council, there were only two employees in the Unit. Three new people have been added since, a GS-2 stay-in-school, a GS-3 WAE and a GS-4 permanent part-time. During the periods of personnel shortage, much overtime was worked to accomplish the mission of the Unit.

The Council workload was reorganized during this fiscal year, resulting in a doubling of effort for the RSU, but the Council members welcomed the change because they had a lesser load to carry. In addition, a rearrangement of the summary statements in the Council books resulted in a more orderly review of the summary statements by the Council member, i.e., they are now divided according to funding potential.

It has been proposed that the September/October Council be held in Hamilton, Montana. Because only the head has been permitted to go from RSU, everyone present including professionals will have to assist in the logistics of the Council meeting.

F. Clinical Trials

This year the updating of 1979 NIAID clinical trials went relatively smoothly, primarily because the Program Analysis Section was back in the business of screening grant applications for clinical trials. A total of 106 clinical trials for 1979 were reported, involving 49 grants (70 trials) 23 contracts (33 trials) and three intramural projects (3.6 man years) for an estimated amount of $6,452,875. (See Table VI)

TABLE VI

NIAID FY-79 CLINICAL TRIALS

CENTERS (P50)

BR	COMPETING # GTS	COMPETING # TRLS	COMPETING FY 79	NON-COMPETING # GTS	NON-COMPETING # TRLS	NON-COMPETING FY 79
01	2	2	$105,537	5	15	$309,388
30						
32						
53						
54						
55						
56						
63						
70						
90						
Total	2	2	$105,537	5	15	$309,388

R01, P01, R22, R23

BR	COMPETING # GTS	COMPETING # TRLS	COMPETING FY 79	NON-COMPETING # GTS	NON-COMPETING # TRLS	NON-COMPETING FY 79
01	4	4	$ 264,779	11	28	$ 441,345
30	3	3	192,243	8	10	514,792
32	2	2	47,024	2	2	77,825
53	0	0	0	1	1	28,203
54	2	2	85,258	0	0	0
55	0	0	0	1	1	55.800
56	3	5	250,993	0	0	0
63	0	0	0	1	1	17,479
70	1	1	18,000	0	0	0
90	3	3	110,210*	0	0	0
Total	18	20	$ 968,717	24	43	$1,135,444

Grand Total	#	# Clinical Trials	Amount
Grants	49	70	$2,519,086
Contracts	23	33	3,699,789
Intramural		3**	234,000
		106	$6,452,875

(cont'd)

* See next page
** See next page

* This amount is lower than that originally submitted to the NIAID Budget office on 5/5/80. Subsequent contact by Dr. Beck with the program directors on two of the three ICIDRs indicated that the trials they described in the application will not be started in FY 79. Therefore, we have assigned "0" dollars for those two trials.

** 3.6 Man Years x $65,000.

Revised 7/17/80

V. RESEARCH RESOURCES BRANCH

A. Special Activities

American Society for Microbiology Exhibit - The Research Resources Branch (RRB) handled arrangements for an NIAID exhibit at the American Society for Microbiology (ASM) meeting held May 11-15, 1980, in Miami, Florida. The RRB assumed overall responsibility for the arrangements in collaboration with the Office of Research Reporting and Public Response. Manpower used during the exhibit hours was supplied by RRB and the Office of Research Reporting with additional assistance volunteered from the Microbiology and Infectious Diseases Program (MIDP). The exhibit was a modification of the one previously shown at the ASM meeting and included a video recording showing and describing the various Institute activities. Although the exhibit gave the visitors knowledge of the various Institute activities, it was not as successful this year as in previous years. This assessment is probably attributable to the following: 1) the overall attendance at ASM was lower than in past years, 2) the NIAID exhibit was not delivered on schedule and the first day of the show was conducted utilizing a table, a television screen and the hand-out materials, 3) the location of the meeting this year was a desirable one for participants to enjoy the many other activities offered in the resort city in lieu of walking through the exhibit hall. Many of the other exhibitors expressed the same opinions.

Dr. Robert Byrne continued to serve as Branch Chief until May 20, 1980 at which time Sylvia Cunningham was appointed Acting Chief. In addition to the Chief, the RRB is supported by one clerical position. The RRB is responsible for the management of operations including distribution and cataloging of the microbial, allergen and some of the immunologic reagents.

The need for well characterized reference reagents as an adjenct to research is well recognized, and in support of the concept, the RRB conducts a program which distributes the reagent with information and technical advice on reagent characteristics and use. The RRB is also sponsoring a contract for the development of a procedure for the processing and packaging of recombinant DNA Vector-Host systems. In addition, the RRB has been assisting with the contract for the establishment of a Plasmid Reference Center.

B. Asthma and Allergic Diseases Program - Allergen Reagents

Under a procurement contract with the American Type Culture Collection, the RRB has been responsible for the processing and packaging of the penicillin minor determinant products which are being used by six NIAID contractors to perform clinical studies to ascertain the penicillin sensitivity of patients. In addition to packaging, the RRB also made arrangements to have the safety testing conducted on the products, procured product labels, prepared package inserts and shipped the materials for testing to the six contractors. Staff also participated in meetings with the Bureau of Biologics, Food and Drug Administration to resolve certain Investigational New Drug (IND) issues relating to the role the RRB played in this project.

The contract with the University of Pennsylvania, on which Dr. Byrne served as co-project officer, was terminated before its expiration because the contractor was unable to produce dogs with well-defined allergy to ragweed pollen.

In addition to the activities listed above, the RRB has continued to distribute allergenic products such as ragweed antigen E, K, and RA3; ryegrass I, II, and III and venoms of Honey bees, yellow hornets, white-faced hornets, yellow jackets, paper wasps and hymenoptera venom diluent; these venom products are available for distribution under an IND, as well as for in vitro use.

C. Immunological Reagents and Resources

The contract awarded in FY 1978 to Flow Laboratories for the Maintenance and Breeding of Rabbits of Known Genotype for use in Immunological Studies has continued to serve a useful purpose in maintaining a colony of 500 specially bred rabbits for use in NIAID intramural immunological studies. In addition, these facilities provide reference quantities of reagents and limited numbers of rabbits to responsible investigators doing immunological studies. During this period, 5 rabbits were provided to an investigator in Alabama and 15 shipments of rabbit antisera have also been made to other investigators.

During this period, the rabbits were moved from their temporary location in Fairfax, Virginia to their permanent quarters in McLean, Virginia. The new facility in McLean is a new animal building which had to be built as a result of the requirement by Metro to have the site of the old building located in Rockville, Maryland.

D. Microbiology

During the latter part of FY 1977, two new contracts were awarded and have continued during FY 1980. The University of Alabama is performing research and developmental work to determine optimal conditions for the preservation, retrieval, storage and distribution of fragile bacterial host strains. Three basic methods of storing have been studied, i.e., preservation in 1) paraffin-sealed agar stabs, 2) by freezing in peptone-glycerol broth and 3) by lyophilization. Results to date indicate that lyophilized material stores better at -30°C or -70°C than at 4°C or room temperature. The peptone-glycerol broth freezing method appears to cause some reversion frequencies of some products.

Studies have been conducted on the lyophilized materials to determine if the reversion frequencies are also occurring in these samples. The MIDP is sponsoring the Stanford University contract for the establishment of a plasmid reference center. The RRB is assisting by storing a back-up set of plasmid strains collected by the contractor.

E. Research Resources - Research Reagents

While responsibility for the actual production of reagents is with the various Institute program areas, RRB has continued to sponsor certain service type contracts which serve the needs of the various program elements.

The Ohio State University continued to serve as the viral reagent testing laboratory until April 15, 1980, at which time the contract expired. While the contract was in effect the contractor completed cross neutralization testing on adenovirus types 10 and 13. This testing was conducted in response to a reagent users observation that the two types might have been misidentified or mislabeled. Ohio State University was unable to confirm the information obtained by the investigator.

After a category of reagents has become established, and the area of research that it supports is well defined, it is of questionable value for RRB to continue storing and distributing that category of reagents for a prolonged period of time. During FY 1975, it was determined that four viral reagent groups fall into this category. The RRB therefore, entered into a five year contractual arrangement with the American Type Culture Collection (ATCC) to transfer the enterovirus, adenovirus, rhinoviruses and arboviruses reagent collections to the ATCC whose prime function is to store and distribute these types of reagents.

Under the contractual arrangement with ATCC, samples of each reagent type will be preserved as a museum function. The viability or homologous antibody activity of a parallel collection of reagents will also be assayed. After each viral group has been assayed and the results reported and reviewed, RRB will discontinue that reagent group from its catalog with a notation that they are available from ATCC and all remaining stock of that group of reagents will be transferred to ATCC for its custodianship. NIH scientists and NIAID contractors will be provided reagents without cost until the RRB supply is depleted.

During the first four years of this project, 50 samples of each reagent type for the four viral reagents groups have been transferred to ATCC for the museum function. The assays of the enterovirus, adenovirus and rhinovirus groups have been completed; these three groups have now been transferred to the ATCC. During FY 1979 and FY 1980 ATCC has primarily been involved in the testing of the arbovirus reagent collection which is the last of the four collections to be re-assayed before being transferred to ATCC. The schedule for assaying and transferring reagents originally prepared in 1975 is still projected; it is planned that the assay of arbovirus reagents will be completed during FY 1980 with the inventory of arbovirus reagents being transferred to ATCC upon completion of review of the data. The transfer is expected to take place during August 1980.

The RRB has also been involved in the labeling and packaging of rotavirus reagents. This project has now been completed and reagents have been distributed to the World Health Organization (WHO) and other investigators.

The intensive work of prior years has resulted in the completion of work on the enterovirus, adenovirus, rhinovirus, myxovirus and the agents and antigens of hepatitis A and B. In most cases, seed virus preparations and corresponding antisera are now available for most of the viruses of public health interest. The reagents which have resulted from the various projects have been most useful, particularly the hemagglutinins, neuraminidases, ribonucleoproteins and the matrix proteins of the influenza viruses of man and animals.

F. Molecular Virology and Immunology Program

The contractor, Georgetown University, is currently evaluating different formulations of HBsAg vaccine and inoculation schedules to optimize the humoral anti-HBs response in adult volunteers. Four sublots of the original HBsAg/adw vaccine were compared using a three dose (40 ug) regimen (zero time, six weeks and six months). The untreated aqueous (Lot A-2) and untreated alum-adsorbed (Lot A-3) vaccines were superior to the ether-tween 80 treated preparations (Lots A-4 and A-5) and vaccination of additional volunteers with progressively smaller doses of the A-2 and A-3 lots was initiated to determine the end-points of the immunogenicity of these two vaccines. Over 50% of volunteers given a single 40, 20, or 10 ug dose of Lot A-2 developed anti-HBs within three weeks of vaccination; seroconversion rates approached 90% by ten weeks following the six week booster injection. With Lot A-3, 80% of vaccinees receiving 40, 20 or 10 ug doses developed anti-HBs within three weeks and 94% by ten weeks following the six week boost. To date, the frequency of seroconversions occurring at each of the three dose levels has been approximately the same for both vaccines; data from groups receiving the 5 ug dose are not yet complete. The alum-adsorbed vaccines elicited a brisk humoral response in adult volunteers (approximately 70% seroconversion in vaccinees within three weeks postinoculation). Such a rapid immune response may be crucial for protection in certain high risk groups such as neonates born of HBsAg-positive mothers and individuals exposed to HBV by accidental needlestick. A final lot of alum-adsorbed vaccine (Lot A-9) and placebo (Lot P-3) were prepared and have passed final vial safety tests. The Lot A-9 vaccine is being considered for prospective studies in children and, possibly, infants subject to the development of suitable protocols.

The delta (δ) antigen and antibody (anti-δ) are new reactivities detected in carriers of HBsAg. Inoculation of HBsAg-positive sera from patients with chronic liver disease and intrahepatic δ into chimpanzees susceptible to infection with HBV resulted in type B hepatitis and markers (δ/anti-δ) in recipient animals. A small RNA of approximately 5 x 10 M.W. was temporally associated with δ in the sera of infected chimpanzees and copurified with the δ-associated particles. This RNA is smaller than the genomes of known RNA viruses but larger than the viroids of higher plants. Delta appears to represent a marker of a transmissible pathogenic agent, either an HBV variant or another agent that requires the helper functions of HBV, that is defective, and that interferes with HBV replication.

The contractor has cloned and amplified HBV full-length DNA and DNA fragments in E. coli using pBR322 and pBR325 vectors. HBV-DNA was radiolabelled by nicked-translation and used as a probe for HBV-DNA sequences in clinical material (tissue and serum). Liver tissue from well-documented cases of HBsAg carriers with hepatocellular carcinoma from Taiwan was examined for HBV-DNA; HBV-DNA sequences were found in both hepatomatous and non-tumor regions of livers from several patients and apparently integrated into the host DNA.

The Hepatitis Testing Laboratory provides support to the various hepatitis programs of the NIAID. The laboratory is currently providing tests for HBsAg, anti-HBs, anti-HBc, anti-HAV, anti HAV IgM, DNA polymerase, HBsAg and anti-HBs d or y subtypes.

A microtiter plate solid phase radioimmunoassay system was developed to detect mouse antibody directed against cells infected with Respiratory Syncytial Virus (RSV). The assay system greatly facilitated the search for mouse myeloma (NS-1) X Balb/c spleen cell hybridoma clones, derived from polyethylene glycol mediated somatic cell fusion, which produce monoclonal antibodies directed against viral components in the initial priming immunogen (HeLa Ohio cells infected with RSV and fixed with 0.2% glutaraldehyde after an 18 hr. infection).

G. Processing and Distribution

In addition to the various program elements detailed above, the RRB also distributes coronaviruses, herpes viruses, interferons, mycoplasmas and reoviruses.

Due to the limited number of reagents to be processed during FY 1980, there was not a formal research and development contract to handle this activity. However, the ATCC did process and package the penicillin products and rotavirus reagents by the purchase order mechanism.

The RRB reagent collection (exclusive of the viral reagents transferred to ATCC) now consists of over 650 individual reagents. The repository and distribution contract remains at Flow Laboratories, McLean, Virginia. A tabular record of distribution by this facility since FY 1972 follows:

DISTRIBUTION OF VIRAL, MYCOPLASMAL, AND ALLERGEN REAGENTS

Fiscal Year	Total Transactions	Total ampoules & vials distributed
1972	572	21,801
1973	575	19,181
1974	500	9,932
1975	592	6,751
1976	762	10,188
1976 (TQ)	192	3,126
1977	613	7,633
1978	605	6,851
1979	782	16,223
1980 (3/4 year)	598	7,305

In addition to the shipments tabulated above, Flow has also made 130 shipments for the Development and Applications Branch, 20 shipments consisting of whole rabbits or rabbit serum and 14 interferon shipments for the National Cancer Institute. All of this was conducted under the NIAID contract with the NCI contributing funds to NIAID for their activities. In addition, the ATCC has made the following shipments under the reagent transfer contract:

(U.S. Investigators and WHO Laboratories)

Fiscal Year	No. of Shipments	No. of Ampoules
FY 1978	96	1,622
FY 1979	110	1,329
FY 1980	74	714

The RRB has continued its efforts to publicize the availability of these valuable reagents to the scientific community. The availability of reagents was incorporated into the NIAID exhibit which was shown at the American Society for Microbiology meeting held in Miami, Florida.

H. Individual Contract and Agreement Tabulation

PROGRAM: IMMUNE SYSTEM AND DISEASE
Research Reagents Branch

Principal Investigator Contract Number Contractor FY 1980 Obligations	Objectives and Findings
Sullivan, Rudolph R. AI 82565 Flow Laboratories $168,219	To maintain a genetically bred rabbit colony of known genotype for use in immunologic investigations. A maximum of 500 adult rabbits will be bred and maintained by expert veterinary care. This is for support of high priority research on genetic control of the immune response and to supply reagents and rabbits to other investigators.

PROGRAM: INFECTIONS - VIRAL REAGENTS,
Research Reagents Branch

Principal Investigator Contract Number Contractor FY 1980 Obligations	Objectives and Findings
Hughes, John R. AI 62513 Ohio State University $17,313 (FY 1978 Funds)	This laboratory serves as a shelf-life viral reagents testing laboratory for members of the enteroviruses, rhinovirus, adenovirus, myxovirus and herpesvirus groups. This contract will ensure the integrity of these reference standard reagents by assessing the potency and purity of viral reagents after long term storage. This contract expired April 14, 1980.

PROGRAM: INFECTIONS - MOLECULAR VIROLOGY AND IMMUNOLOGY
Research Reagents Branch

Principal Investigator Contract Number Contractor FY 1980 Obligations	Objectives and Findings
Gerin, John AI 82572 Georgetown University $945,998	During FY 1980 the laboratory was involved in evaluating different formulations of HBsAg vaccine and inoculation schedules to optimize the humoral anti-HBs response in adult volunteers. A new lot of vaccine is currently undergoing final vial safety testing and is being considered for prospective studies in children and, possibly infants subject to the development of suitable protolols. The contractor has cloned and amplified HBV full-length DNA and DNA fragments in E. Coli using pBR322 and pBR325 vectors. HBV-DNA sequences in clinical material (tissue and serum). Liver tissue from well-documented cases of HBsAg carriers with hepatocellular carcinoma from Taiwan was examined for HBV-DNA; HBV-DNA sequences were found in both hepatomatous and non-tumor regions of livers from several patients and apparently integrated into the host DNA. The Hepatitis Testing Laboratory provides support to the various hepatitis programs of the NIAID. The laboratory is currently providing tests for HBsAg, anti-HBs, anti-HBc, anti-HAV, anti-HAV IgM, DNA polymerase, HBsAg and anti-HBs d or y subtypes. A microtiter plate solid phase radioimmunoassay system was developed to detect mouse antibody directed against cells infected with Respiratory Syncytial Virus (RSV). The assay system greatly facilitated the search

for mouse myeloma (NS-1) X Balb/c spleen cell hybridoma clones, derived from polyethylene glycol mediated somatic cell fusion, which produce monoclonal antibodies directed against viral components in the initial priming immunogen (HeLa Ohio cells infected with RSV and fixed with 0.2% glutaraldehyde after an 18 hr. infection).

Individual Contract Agreement Tabulation

PROGRAM: INFECTION - PROCESSING AND DISTRIBUTION
Research Resources Branch

Principal Investigator Contract Number Contractor FY 1980 Obligations	Objectives and Findings
Stanley, Thomas AI 82542 Flow Laboratories $119,861 (MIDP) $267,443 (IAIDP)	When the reagents program was given the responsibility for production of reference reagents, other responsibilities were also included, i.e., the development and maintenance of appropriate means to receive, process, store and distribute these research materials. The contractor fulfills these responsibilities. During the past year, the contractor has been supplying the storage and shipping services for the RRB, Development and Applications Branch, the Enteric Diseases Branch and the NCI. As a result of these combined activities the contractor has made 2,548 shipments totaling 106,614 vials plus 38,670 trays and 570 shipments of bulk material. Approximately 95 incoming shipments have been handled.

PROGRAM: INFECTIONS - PROCESSING AND DISTRIBUTION
Research Resources Branch

Principal Investigator Contract Number Contractor FY 1980 Obligations	Objectives and Findings
Clark-Curtiss, Josephine AI 72533 University of Alabama $171,657 (FY 1977 funds) Contract awarded for a 3 year period	This contractor is determining the optimal processing, packaging and storage conditions for vector-host systems for recombinant DNA molecule research. To date, the contractor has investigated various methods of preserving these materials. The methods employed to date include: 1) preservation in paraffin-sealed gelatin slabs, 2) preservation by freezing in peptone - glycerol, preservation by lyophilization. In addition, a study is being conducted comparing the effects of various storage temperatures.

PROGRAM: INFECTIONS - PROCESSING AND DISTRIBUTION
Research Resources Branch

Principal Investigator Contract Number Contractor FY 1980 Obligations	Objectives and Findings
Donovick, Richard AI 52526 American Type Culture Collection $149,823 (FY 1979 funds)	After a category of reagents has become established and the area of research that it supports is well defined, it is of questionable value for RRB to continue storing and distributing that category of reagents. Therefore, in FY 1975, the RRB entered into a cooperative enterprise with the ATCC to transfer the enterovirus, arbovirus, rhinovirus and adenovirus reagent collections to the ATCC for their custodianship and distribution; complete transfer of the four viral reagent groups will be accomplished over a five-year period. During the first three years of the contract, 50 samples of each reagent type for the four viral reagent groups have been transferred for museum storage purposes. Under the terms of the contract, each of the four collections of reagents is to be assayed for viability or homologous antibody before the complete transfer is made, therefore, the contractor has been assaying the enterovirus, adenovirus, rhinovirus groups during the initial period. The assay of these groups has been completed, the test results have been reviewed, and the total enterovirus, adenovirus, rhinovirus collections have been transferred to ATCC. The contractor is now performing the required testing on the arbovirus reagents. The contract terminated July 15, 1980.

I. Contract Publications

Hess, G., J. W.-K. Shih, W. Arnold, J. L. Gerin and K.-H. Meyer zum Buschenfelde. 1979. Demonstration and partial characterization of the 22-nm HBsAg and Dane particles of subtype HBsAg/ady. J. Immunol. 123:1189-1194.

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OFFICE OF THE SCIENTIFIC DIRECTOR, NIAID

1980 Annual Report

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SUMMARY OF PROGRAM

Laboratory and Clinical Research, NIAID

October 1, 1979 through October 1, 1980

Office of the Scientific Director

The Annual Report of the Intramural Research Program contains individual summaries of research projects in the twelve laboratories which constitute the research component of the National Institute of Allergy and Infectious Diseases. Administrative responsibility for the Intramural Research Program resides in the Office of the Scientific Director (OSD). The administrative functions of the OSD continue to remain the responsibility of Mr. Charles Criswell at the Bethesda campus and Mr. Robert Steiner at the Rocky Mountain Laboratory. Major responsibility for all intramural contracts was assumed by the Assistant Scientific Director, Dr. Gordon Wallace. The contract program has been redeveloped and redesigned to ensure its compliance with all contract program requirements and to provide maximum support services to individual laboratories at NIAID. During the past year, new contracts were initiated (a) to support the development of hybridomas for immunogenetics research (b) to study the basic biochemistry of poly ICLC and interferon inducers which have been developed by Dr. Hilton Levy of IRP and (c) a new contract for the performance of clinical and immunological studies in support of the Laboratory of Clinical Investigation. Some contracts from previous years also continued to receive support. Support of the Pacific Research Unit is in its final year.

Recruitment of personnel for intramural research laboratories remained a major responsibility and a high priority for the OSD. One major accomplishment was the recruitment of Dr. John Swanson, Professor of Pathology from the University of Utah, who assumed the role of Chief of the Laboratory of Microbial Structure and Function at the Rocky Mountain Laboratory in October of 1979. He immediately began to restructure the Laboratory and recruit new personnel. Dr. Bruce Chesebro was selected to serve as the Chief of the Laboratory of Persistent Viral Diseases. The third "new" Laboratory at the Rocky Mountain Laboratory is currently headed by Dr. Robert Philip who has agreed to serve as Acting Chief until a permanent Chief can be selected. Recruitment activities continue in order to find a Chief of equal scientific stature and quality as of those already selected for the other two Laboratories at RML. It is the intent of OSD and NIAID to develop a scientifically sound, well-rounded, and intellectually competitive environment for RML.

In order to increase scientific communication at the Rocky Mountain Laboratory in Montana, we have supported the exchange visit between scientists at the NIAID Bethesda campus and those at RML. In addition, national and international meetings have been held at RML, including a meeting on arbovirus disease, and a meeting on rickettsial diseases. In addition, a two-volume book on slow virus diseases was published as the result of an international meeting on slow virus diseases which was held at RML during the previous year. The Council of the National Institute of Allergy and Infectious Diseases was convened at the RML in late September, 1980, in order to provide them with a direct view of the changes which are now taking place at the Rocky Mountain Laboratory. To implement the reorganization at RML, it was necessary to undertake many major renovations. The media and glassware section located in Building 3 was relocated to Building 10. The renovation of Building 10 and the first floor of Building 3 was undertaken by the local work force of RML. Mr. Robert Steiner, the Chief of the Operations Branch, as well as the members of the RML shop services, deserve special credit for their work. They received a cash award for their efforts.

In order to provide for better communications between RML and the Bethesda campus, dedicated telephone lines were installed which allow for the use of speaker-telephones as well as a "magic" blackboard so that Lab Chiefs' meetings and other administrative meetings can be held jointly at Bethesda and RML. In addition, a new Rapifax fascimile transmitter was installed so that all documentation transmitted between each of the facilities could be sent and received on the same day. It is anticipated that these facilities will be used for joint scientific meetings in the future.

At the Bethesda campus, we have expanded the activities of the Editorial Office, and six laboratories are now served by the Office which has four editorial assistants. The Head of the Editorial Office, Betty Sylvester, has been active in developing a very competent and responsive program which has been generally accepted and praised by all scientists who have an opportunity to use the capabilities of the Editorial Office. This office provides not only for editorial review of manuscripts and their prompt completion, using the most recent System Six IBM machines, but also provides for careful analysis of figures, tables;

and a library investigation of bibliographical references to ensure accuracy. The quality of publications, as well as the ease with which they are prepared, has improved enormously in the past year because of the efforts of the Editorial Office.

We continue to seek ways to recruit minority scientists for the IRP/NIAID. For the second successive year we held a Minorities Biomedical Science Program, during which 48 students were invited to visit NIH and spend three days with scientists in their laboratories, learning of work opportunities. A total of 15 were offered summer positions in 1980. Dr. Cook Jaouni provided the leadership and management of this program with outstanding results. The students selected in this program provided the base for minority applicants for the summer program which in the summer of 1980 totaled 95 students, of whom 41 were female. Of these 95, three were native Americans, seven were Asian, 29 were black and 14 were Hispanic.

After reviewing the hiring, promotion and training figures for NIAID, it seems clear that we need to set priorities for our EEO program. There is a particular shortage of Hispanic and native American employees at all grade levels and in all positions at NIAID. There is also a shortage of all minorities and women in Grades GS-13 and above. It is quite clear that the solutions to these two problems are quite separate. Those personnel of GS-13 and above are most commonly research scientists, and this recruitment must be undertaken in professional schools using the same criteria for quality standards that we have always expected for our science program at NIAID. Gauging from the success of our summer program and our ability to find extremely highly-qualified minority students, it would seem that these may ultimately be the source of both minority and women candidates for our post-doctoral and scientific positions in years to come.

The Animal Care Section and its facilities continue to be a center of great activity in IRP. It is the goal of OSD to have the facilities and operation of the NIAID Animal Section in full conformity with the American Association of Laboratory Animal Accreditation. We have just completed a total renovation of the animal facilities in Building 7, making them fully accreditable for animal care. We have also upgraded the facilities on the 11th floor of Building 10 by the installation of chilling/cooling units and by the use of laminar flow facilities in order to provide the necessary air changes to meet accreditation standards. The last animal area to be renovated will be in Building 5.

Two new animal care contracts were initiated during the year-one for the breeding and one for the holding of mice. These contracts are located in facilities which are especially designed to provide for optimum care to meet AALAC standards. The breeding contract will hold up to 7,000 mice and the holding contract will house between 5 and 10,000 mice, thus expanding our capability to handle experimental animals or, more importantly, to handle rodents under optimum conditions of care. We have also hired an expert consultant, Dr. Peter Golway, to serve as veternarian for OSD and provide the necessary professional evaluation, modification and input to both facilities and training programs for the animal section at our NIAID. Dr. Golway has an excellent background in small animal care and is especially interested in the re-structuring and upgrading of the NIAID facilities.

The Intramural Program of NIAID also continues to have a major commitment to the Risk Assessment Program for Recombinant DNA research. During the past year, Dr. Malcolm Martin and Dr. Wallace Rowe, have expanded their studies of polyoma plasmid expression in E. coli to further substantiate their early work showing the safety of this system. They have also expanded their work by the initiation of marine leukemia-plasmid-E. coli experiments in order to explore other viral pathogens. The NIH Safety Branch was instrumental in gaining approval from the recombinant DNA Advisory Committee for the conduct of experiments which have previously been prohibited. This would allow the Intramural Program of NIAID to undertake cloning experiments using vectors transmission into wild-type E. coli under P4 conditions. Such experiments are now being discussed and planned and probably will be initiated in fiscal '81. Intramural scientists were also active participants in discussions of the potential risk of auto-immunity from bacteria-producing hormones which might cause autostimulation.

During the past year NIH has been actively involved in the development of a program for the "round robin" renovation of the older buildings on the NIH campus. Initially, they suggested the evacuation of Building 5 which would then serve as a place where renovations would begin. This would have caused a major dislocation of NIAID scientists for as many as ten or twelve years until the complete round robin of renovations had been completed. A plan was worked out with the Deputy Director for Science and the Long-range Planning Committee of NIH, which would allow for the relocation of personnel in Building 8, so that the renovations could begin with this Building. The advantages of this were as follows: NIAID personnel from Building 8 could be moved to Building 5 into spaces that would be vacated by the movement of animals from Building 5 to Building 14B (Veterinary Resources Branch). Building 8 then could be renovated and, at the same time, expanded by the addition of one floor and an extension of 50 feet on to one end of the building so that it would then provide 40,000 sq.ft. of laboratory space, a space equal to the largest of the old research buildings(that is, Buildings 4 and 5). This would allow the occupants of Building 4 to then move to Building 8. Then Building 4 would be renovated to meet the needs of our NIAID occupants of Building 5. While the time table is not yet firm, it would appear that animals currently located in Building 5 would be relocated to Buildings 14B during 1981 or 1982, and the scientists and laboratory occupants of Building 8 will then move to spaces in Building 5 in 1982-83. It would be some four years later, or approximately January 1987, before Building 4 would then be available for the occupants of Building 5.

It should be noted also that, at the time of the initial discussions of the "round robin" renovations, it had been thought that Building 7 should be demolished. Our recent efforts to make Building 7 more habitable have been well received, and it is now included in the round robin renovation, probably as the last building to be renovated.

Safety continues to have high priority in IRP/NIAID. Building 7 was considered the most unsafe building occupied by NIAID up until approximately one year ago. At that time, we initiated a contract for the relocation of most of the freezers which occupied the hallways of Building 7. With their relocation and the removal of other equipment from the hallways of Building 7, we now are nearly in full compliance with fire code and safety requirements for this building. We now intend to use the freezer contract to relocate freezers occupied in the hallways of Building 5 and the 11th floor of Building 10 to improve the hallway safety and safe egress from those areas as well. We continue to respond promptly to safety surveys being conducted by the Safety Branch and have made a special effort to respond to the new environmental protection agency requirements for toxic waste disposal. At NIH this is primarily taken care of in the Safety Office. However, at the Rocky Mountain Laboratory, we have direct responsibility for toxic waste removal. Two members of the NIH/EPA team and Dr. Katherine Cook Jaouni, Intramural Safety Officer, visited the Rocky Mountain Laboratory in August of 1980, to evaluate the conditions there and to prepare the initial report which must be submitted to EPA. During the year we also had installed and certified a P3 level laboratory at RML for the conduct of Q fever experiments.

For some time, OSD has been aware of a need for evaluation of intramural supervisors in order to provide for appropriate training in all of their responsibilities. In August of 1980, a contract was awarded which would provide for an analysis of the capabilities and knowledge of intramural supervisors coupled with an evaluation of our administrative operation of the Intramural Program. The contractors were to take special note of the dual system of management afforded by the (a) Health Science Management of the Scientific Personnel (Laboratory Chiefs) and (b) the Administrative Offices.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AI-00013-17 OSD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies of Viral Antigens in Virus-induced Tumors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI	Andrew M. Lewis, Jr.	OSD, NIAID
Other	Hubert W. Gerry	Staff Fellow, NIAID

COOPERATING UNITS (if any) Arthur Levine, Cephas Patch, Alan Rabson (NCI); Heiner Westphal, (NICHHD) Robert Martin (NIAAMD)
James L. Cook, John B. Hibbs, University of Utah Medical Center

LAB/BRANCH
Office of the Scientific Director

SECTION

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.0	2.0	3.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The initial objectives of this project were to use human adenoviruses and adenovirus-SV40 (Ad2-SV40) recombinants as tools to study the genetics of DNA tumor viruses, to define the role of viral genes and viral antigens in viral oncogenesis and to study the biology of Ad2-SV40 recombinants. Due to the lack of proper containment facilities in NIAID between January 1973 and May 1978, there was a 5-year disruption(see past repports) in the major thrust of this project. During this interval, a study of Ad2-SV40 recombinants associated tumor induction in Simian hamsters by these agents with the incorporation into the adenovirus-2 chromosome of a specific segment of SV40 DNA. To begin to understand the mechanisms by which this SV40 DNA segment conveys oncogenicity to these recombinants, it was necessary to understand why Ad2 was nononcogenic and why SV40 was highly oncogenic for hamsters. Since Ad2-SV40 will transform hamster cells in tissue culture, we initiated a study of the oncogenic properties of cells transformed by these viruses. The results thus far lead us to believe that SV40 conveys to transformed hamster cells the ability to resist rejection by the host's cellular immune system. Conversely, adenovirus 2 appears to be unable to convey resistance to cellular immune re-

jection to the cells it transforms. These data imply that, in addition to transformation, the induction of some type of resistance to cellular immune rejection is essential to the process by which SV40 converts normal cells to tumor cells. The incorporation of the segment of the SV40 genome which induces this resistance to cellular immune rejection into the adenovirus 2 chromosome could explain the mechanism by which SV40 conveys oncogenicity to certain adenovirus 2-SV40 recombinants.

Major Findings

The discovery of allograft-rejection resistance in SV40 transformed hamster cells - its possible role in tumor development.

SV40 is an oncogenic DNA virus that induces tumors in vivo when inoculated into rodents and transforms cells in vitro from a variety of species, including rodents, primates, and humans. Tumor induction by SV40 appears to be unique to Syrian hamsters, mastomys, and certain strains of mice because no virus-induced tumors have been observed in rats, guinea pigs, rabbits, monkeys, and humans inoculated with this virus. With the exception of cells from Syrian hamsters, cells transformed in vitro by SV40 usually do not produce tumors when inoculated into autologous or isologous hosts; however, such nononcogenic, SV40-transformed cells will frequently grow as tumors in immunologically compromised animals. Thus, the oncogenicity of SV40 is species specific, and the lack of oncogenicity of SV40-transformed cells for an appropriate host appears to be due to the inability of these cells to escape the immunological responses of the recipient. Available data imply that the early SV40 proteins (encoded prior to viral DNA synthesis) are responsible for the transformed state that, in turn, results in tumor development in hamsters. However, the lack of oncogenicity of SV40 for most other species and the lack of oncogenicity of SV40-transformed cells containing the early SV40 proteins suggest that species-specific factors restricted to hamsters are also involved in SV40's tumor-inducing capacity. To explain the susceptibility of hamsters to SV40 tumor induction, it has been generally assumed that these animals are relatively immunoincompetent compared to other mammals. However, recent data suggest that the immunological responsiveness of hamsters is not significantly different from that of other species. These data include the expression of strong cellular and humoral alloimmune reactions and the thymus-dependent rejection of Ad2-transformed cells. Another explanfor the species-specific oncogenicity of SV40 and Sv40-transformed cells is that SV40 might induce a unique change during transformation of hamster cells that renders the resulting neoplastic cells resistant to host rejection. By studying the efficiency of tumor induction by SV40-and Ad2-transformed inbred hamster cells and transformed cell-induced tumors, we have developed data that support this possibility. Four clonally derived SV40 transformed inbred hamster (LSH strain) embryo cell lines induced tumors with equal efficiency in both syngeneic adult LSH and allogenic (as shown by skin graft rejection, mixed lymphocyte reaction, and graft versus host reaction) CB hamsters. The average number of SV40 transformed cells per tumor producing dose at the 50% endpoint (TPD_{50}) for LSH hamsters was $10^{4.0}$ while the average TPD_{50} for CB hamsters was $10^{4.9}$--a difference that was not statistically significant.

Three Ad2 transformed LSH embryo cell lines which induced tumors in newborn hamsters,failed to induce tumors in either syngeneic LSH or allogeneic CB adults (TPD_{50}'s > 8.3). Seven SV40 transformed cell-induced tumors also produced tumors with equal efficiency in both syngeneic and allogeneic hamsters; the TPD_{50} LSH/TPD_{50} CB varying only 5 fold. In contrast, 4 syngeneic adult adapted (by serial passage in newborns)2Ad transformed cell-induced tumors produced tumors efficiently only in syngeneic LSH adults; the TPD_{50} LSH/TPD_{50} CB vary> 1,000 fold.

These results show that SV40, in contrast to Ad2, conveys what appears to be a species-specific resistance to allograft rejection to transformed Syrian hamster cells and imply that, in addition to transformation, the induction of some type of resistance to rejection by the cell-mediated responses of the host is necessary to the process by which SV40 (and possibly other tumor viruses) convert normal cells to tumor cells.

The thymus-dependent rejection of Ad2 transformed cells by Syrian hamsters.

The tumor-inducing capacity of two Ad2 transformed hamster cell-induced lines (Ad2HTL3 and Ad2HTL6) was tested in immunocompromised syngeneic adult hamsters. An average of 35.2% of neonatally thymectomized, syngeneic, weanling hamsters developed progressively enlarging tumors when injected subcutaneously with tumor suspensions prepared from these lines, while no tumors were observed in normal, syngeneic, weanling hamsters challenged with the same inocula. The susceptibility of neonatally thymectomized hamsters to tumor challenge was directly related to the degree of immunosuppression observed following thymectomy as indicated by the amplitude of the in vitro response of whole blood cultures to concanavalin A. Pretreatment of thymectomized weanlings with syngeneic adult lymphoid cells resulted in a significant reduction in tumor susceptibility ($p = 0.03$). These findings suggest that the maturation of a thymus dependent cell-mediated immune response in hamsters during the first 21 days of life results in the rejection of Ad2-transformed cells.

Transplantation of the Ad2HTL6 newborn tumor line to suckling hamsters 1-15 days old demonstrated that maturation events in the hamsters immune system between 6 and 7 days were responsible for tumor rejection. Very little data has been published concerning the ontogeny of the hamster immune response; however, based on data from other species, it has been predicted that hamster thymus and spleen cells should respond to T cell mitogens within a few days of birth and that suckling hamsters should be able to reject skin allografts at 8 days of age. To begin to evaluate the development of cellular immune responses in hamsters, cells from bloods and spleens from different aged animals were tested for their ability to respond to the mitogen conconavalin A (ConA) and to respond in the mixed lymphocyte reaction. The results of these studies indicate that hamster spleen cells begin to respond to ConA by age 5 days and attained 100 percent of adult type responses by age 15 days. Cells in the blood did not respond to ConA until age 25 days.

Hamster spleen cells did not begin to respond in the mixed lymphocyte reaction until 18 to 25 days. To determine if the lack of ConA responsiveness of hamster spleen cells before age 5 days was due to T cell immaturity or immaturity of another cell component which was required for this response, X-ray inactivated adherent and nonadherent (to distinguish between lymphocytes and macrophages) adult peritoneal exudate cells were mixed with spleen cells from 3 day old hamsters and the mixtures tested for their ability to respond to ConA. These results showed that, in the presence of populations of adherent peritoneal exudate cells, 3 day old hamster spleen cells readily responded (stimulation indices of 13 to 35) compared to spleen cells alone (stimulation index of 1 to 2) or spleen cells mixed with nonadherent cells (stimulation index of 1 to 3). These results imply that immaturity of cell populations which cooperate with lymphocytes in the response to ConA are most likely responsible for the apparent immaturity of this reaction in hamsters less than 5 days old.

The resistance of SV40 transformed hamster cells to the cytolytic effect of activated macrophages as a possible factor in tumor induction.

To begin to evaluate the reasons for the apparent resistance of SV40 transformed hamster cells to host cell-mediated defenses, (Cook, Hibbs, Lewis), we have studied the effects of "activated" macrophages from animals with chronic intracellular infections on SV40- and Ad2-transformed rodent cells. The battery of target cells evaluated displayed three different transformed cell phenotypes when inoculated into animals--nononcogenic, oncogenic in the host of origin but not histoincompatible host, and oncogenic in both the host of origin and histoincompatible host--and provided the opportunity to study the relative susceptibilities of these different target cell types to killing by activated macrophages. Activated macrophages are one of the effector cell components of cellular immune reactions that have been shown to inhibit the growth of or to lyse neoplastic cells *in vitro*. The tumoricidal effect of these activated macrophages is mediated by a nonphagocytic mechanism that requires intimate contact with target cells. The results obtained thus far suggests that resistance to the cytolytic effect of tumoricidal, activated macrophages is one of the mechanisms by which SV40-transformed hamster cells may evade destruction by the host.

Immunogenicity of Ad2 transformed hamster cells and transformed cell-induced tumor lines .

To determine the possible role of Ad2 transplantation antigen (TSTA) in the rejection of tumors transplanted to weanling hamsters, several types of studies are underway. An assay has been developed for Ad2 TSTA using an Ad2 tumor line (Ad2HTL1), which had been adapted to grow in adult hamsters. Immunization with Ad2 significantly protected (defined as a resistance index *RI) greater than 10) hamsters against a challenge with Ad2HTL. Immunization with SV40 did not convey significant protection (RI between 1 to 10) in these experiments. This assay is being used to study the immunogenicity of Ad2 transformed cell lines and newborn and weanling tumor lines developed from these transformed cells. Results thus far indicate that 3 Ad2 transformed cell lines (Ad2HE1, Ad2HE3, Ad2HE6) and the tumor lines established from them (Ad2HTL1, Ad2HTL3, Ad2HTL6) contain a common Ad2 TSTA which during immunization with x-irradiated cells conveys protection to hamsters challenged with Ad2HTL1. Differences in RI did not correlate with the differences in the transplantability of the newborn tumor lines to newborn and weanling hamsters. Adult hamsters that had rejected tumors induced by viable Ad2HTL3 and Ad2HTL6 cells were also protected when challenged with Ad2HTL1. These results show that whether they are accepted or rejected by weanling hamsters, each of these lines contains detectable Ad2 TSTA. Such findings appear to exclude the possibility that certain tumor lines can be transplanted to older animals because they lose detectable Ad2 TSTA. To determine whether a reduction in the concentration of Ad2 TSTA is responsible for the transplantability of certain newborn tumor lines to adults, the amount of immunizing antigen in suspensions of different tumors is being evaluated.

Selection of viral progeny with specific DNA modifications involving the loss of RNA splice signals during the replication of the nondefective hybrid Ad2+ND$_4$.

The molecular mechanisms by which animal viruses adapt themselves to replicate in cells from different species and in different cells from the same species are poorly understood. The inherent defenses within a prospective host cell against possible viral intruders are pierced only by those viruses which have mastered the task of altering their gene control mechanisms to meet the requirements of the new host. To expand our understanding of virus replication and virus-host cell interactions, it would be useful to develop model systems to study the ways in which viruses become capable of growing in different hosts. We have found that the nondefective recombinant between adenovirus 2 and SV40, Ad2+ND$_4$, adapts in an unique way to optimal growth in two different host cell species. In monkey cells, viable progeny (capable of independent growth) seem to retain splice signals in their DNA that are recognized by that host. In human cells, viable progeny have lost the DNA region containing these splice signals. The affected DNA sequences are part of the early region of SV40 which is incorporated into the adenovirus 2 chromosomes during the formation of the Ad2+ND$_4$ recombinant. At this time, we speculate that the adaption of this virus to replicate in different host cells may be determined at the level of mRNA maturation. Studies are underway to further define this concept.

Significance to Biomedical Research

Our ability to expand our understanding of infectious agents and the diseases they produce requires the continued development of laboratory models which are amenable to study. The foregoing report documents the progress we have made in developing 2 new model systems which hopefully will provide fundamental insights into 2 important aspects of viral infections, namely, the ability to produce tumors and the capacity of viruses to parasitize cells from different species.

Although many reports have documented the inability of virus-transformed cells to induce tumors in appropriate hosts and the lack of association between cell transformation in tissue culture and tumor induction in animals by oncogenic viruses, there has been no satisfactory explanation for this discrepant behavior. To understand these phenomenon model systems offering possible explanations must be developed and studied. We are optimistic that the Ad2/SV40 transformed hamster-cell system we are studying will be useful in beginning to provide a definitive explanation of the essential factors associated with the conversion of normal cells to tumor cells by oncogenic viruses.

The recently described phenomenon of RNA processing introduces a new level of regulation of molecular events in eukaryotic cells. The influence this regulatory system has on animal virus replication are currently being defined. Our studies thus far suggest that the virus-host cell systems associated with the replication of the nondefective Ad2-SV40 hybrid, Ad2+ND_4, will be useful in studying the effect of RNA processing on the ability of this recombinant to replicate in cells from different species. Information from such studies could be important in assessing and controlling the attenuation of viruses for the development of live virus vaccines.

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MITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AI-00018-14 OSD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Biological and biochemical characterization of human papovaviruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator: Kenneth K. Takemoto, Tatsuo Miyamura, Visiting Fellow, and Kunito Yoshiike, Visiting Scientist - NIAID, OSD.

COOPERATING UNITS (if any)
Peter Howley, Ming-Fan Law, Francoise Rentier-Delrue, LP, NCI; George C. Fareed, Hawley Linke, UCLA, Los Angeles; Mark Israel, NIAID.

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Virology Unit

INSTITUTE AND LOCATION
National Institute of Allergy and Infectious Diseases, Bethesda, Md.

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The human papovavirus JCV which heretofore has been grown only in human fetal glial cells was successfully adapted to growth in readily available human embryonic kidney cells. During the process of adaptation, defective viruses were generated which became the predominant type of virions in the adapted virus pool. The adapted virus formed plaques in HEK cells. However, cloned virus stocks derived after plaque-purification still yielded 2 or more species of viral DNA. The viral DNA was then molecularly cloned and analyzed by restriction endonuclease cleavage and by electron microscopic studies of heteroduplex molecules. From such analysis it was found that 2 kinds of DNA molecules were present in the stock, each with approximately 30% deletions in the early and late regions of the genomes. It is therefore postulated that the HEK adapted virus replicates efficiently in these cells by complementation of defective virions.

PHS-6040
(Rev. 10-76)

Project Description:

JC virus (JCV) replication in human embryonic kidney (HEK) cells. JCV, the papovavirus most frequently associated with the demyelinating disease (PML), is still a poorly understood virus primarily due to its severe host range restriction. Since it grows only in human fetal brain cells, the study of this important virus has been limited to only 1 or 2 laboratories. We have conducted a careful analysis of a number of different kinds of human cells for their ability to support JCV growth and have found that human amnion and kidney cells are permissive for JCV replication. Of the 2 types of cells, HEK cells yielded larger amounts of virus, and enabled the production of substantial quantities of purified JCV. In addition, the HEK-adapted virus produced plaques from which plaque-purified stocks were made. The plaque-purified virus grew much faster in HEK cells than uncloned parental virus and induced viral and tumor antigens much earlier. Cytopathic changes were also evident earlier and were more extensive. The DNA from parental HEK-adapted JCV as well as plaque-purified virus was analyzed by gel electrophoreses after cleavage by restriction endonucleases. The DNA from uncloned virus was heterogeneous; DNAs from plaque-purified virus stocks were less heterogeneous, but still consisted of 2 or more species of viral DNA.

Molecular cloning of JCV DNA from HEK-adapted virus. Since the DNA of the HEK-adapted JCV was heterogeneous even after plaque-purification, it was necessary to obtain molecularly cloned JCV DNA using the pBR322 plasmid vector. The DNA from one of the plaque-purified stocks (Pl. 4) was cloned at either the Eco RI site (0.0 map unit) or the Bam HI site (0.51 map units). The cloned DNA was analyzed by 2 different methods. The first was by restriction endonuclease cleavage followed by gel electrophoresis and the second was by electron microscopy of heteroduplex molecules. By these mehtods, it was found that there were 2 classes of DNA molecules. One had a deletion of 30% between 0.7 and 1.0 map units from the Eco RI site and an insertion of 1% at the same site in the late region of the viral genome. The other class of viral DNA contained a deletion of 35% at map position 0.18 to 0.53 in the early region and also had an insertion of 12% in the early region. In addition, the 2 types of DNA contained added sequences near the origin of DNA replication.

Based upon these analyses, we have concluded that in the process of adaptation of JCV to growth in HEK cells, defective viruses have been generated which replicate by complementation.

Interference between BKV and JCV. The interaction between 2 human papovaviruses BKV and JCV was studied in human embryonic kidney cells. When HEK cells were simultaneously infected with the 2 viruses, marked inhibition of BKV replication was observed by immunofluorescence as well as by infectivity assays. Plaque formation by BKV was reduced by approximately 2 logs in cultures previously or simultaneously infected with JCV. Interference appeared to be highly specific to BKV, since the replication of adenovirus was not inhibited by JCV under similar conditions. Early steps in BKV replication appear to be involved in the phenomenon since BKV T-antigen as well as BKV DNA synthesis were inhibited by co-infection with JCV.

These studies indicate that in their natural hosts, humans; interference between the 2 viruses may occur in organs or tissues where they may co-infect, i.e., the kidneys. These results also suggest that recombination between the 2 viruses cannot occur, which correlates well with the finding that the human papovaviruses are either of the BKV or JCV types.

New lymphotropic papovavirus. The isolation of a new lymphotropic papovavirus was recently reported by investigators in Germany The virus was isolated from lymphocytes of an African green monkey and identified as a new member of the polyomavirus genus. We have begun studies aimed at characterizing the virus biologically and biochemically. Although the natural host of this virus is the African green monkey, an unusual finding is the presence of antibody to the virus in approximately 25% of humans. This is strong evidence that humans are infected with a similar, related lymphotropic virus.

The virus replicates only in monkey and human lymphoblastic cells and only in B and not T cells. It does not hemagglutinate human, mouse, hamster, and sheep erythrocytes.

Preliminary investigations of the genome of this virus have shown that it has a DNA of smaller size than that of SV40.

Significance to Biomedical Research. The papovaviruses represent a class of persistent viruses which have the interesting and important property of establishing chronic infections and also having oncogenic potential. One of the human papovaviruses, JCV, has clearly been demonstrated to be involved in the slow, demyelinating disease, progressive multifocal leukoencephalopathy. Because this virus has been shown to be oncogenic for lower animals including primates and can transform cells in culture, its possible role in other human disease needs to be assessed.

Proposed course. Detailed investigations on the characterization of JCV adapted to growth in human embryonic kidney cells will continue. The molecularly cloned JCV DNAs which have major deletions in the early as well as late regions of the genome will be tested for biological activity. The DNAs will also be sequenced in order to determine the nature of the deleted as well as the inserted sequences.

Biological and biochemical characterization of the new lymphotropic papovavirus will continue.

Publications

Takemoto, K.K. Human polyomaviruses: evaluation of their possible involvement in human cancer. Cold Spring Harbor Symposium on Viruses and naturally occurring cancer. (In Press.)

Miyamura, T. and Takemoto, K.K. Helper function for adenovirus replication in monkey cells by BK human papovavirus. Virology 98, 279-282, 1979.

Israel, M.A., Martin, M.A., Miyamura, T., Takemoto, K.K., Rifkin, D., and Pollack, R. Phenotype of polyoma-induced hamster tumor cell lines. J. Virology, 35, 252-255, 1980.

Miyamura, T., Yoshiike, K., and Takemoto, K.K. Characterization of JC papovavirus adapted to growth in human embryonic kidney cells. J. Virology (In Press.)

Howley, P.M., Rentier-Delrue, F., Heilman, C.A., Law, M-F., Chowdhury, K., and Takemoto, K.K. Cloned human polyomavirus JC DNA can transform human amnion cells. J. Virology. (In Press.)

Awards and Honors:

Speaker, Cold Spring Harbor Symposium on Viruses and Naturally Occurring Cancer, September, 1979. "Human polyomaviruses: evaluation of their possible involvement in human cancer."

Lecturer, Texas Tech University, Department of Biological Sciences, Dec. 4, 1979. "Papovaviruses and Cancer."

Invited lecturer, Hahneman Medical College, Philadelphia, Pa., Feb. 25, 1980. "Recent studies on replication and transformation by human papovaviruses."

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AI-00019-06 OSD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies on the Treatment of Disease with the Interferon System

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI : Hilton B. Levy

*Peter Wiernik, Baltimore Cancer Research Center, Dr. Susan Krown, Sloan Kettering Institute; Dr. Herbert Oetgen, Sloan Kettering Institute; Dr. Martin Lerner, Wayne State University; Dr. Brian Durie, University of Arizona; Dr. Sidney Salman, University of Arizona; Dr. Jordan Gutterman, M. D. Anderson Hospital; Raymond Alexanian, M. D. Anderson Hospital; Dr. Arthur Levine, NCI; and Dr. John Whisnant, University of North Carolina; and Dr. Ed Stephen, USAMRIID, Ft. Detrick.

COOPERATING UNITS (if any) Dr. Beatrice Lampkin, Children's Cancer Study Group, Cincinnati, Ohio; Dr. George Baer, Center for Disease Control, Atlanta, Georgia; Dale Stringfellow, Upjohn Company; Dr. King Engel, Andre Solazar, NINCDS; (see above)*

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SECTION
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INSTITUTE AND LOCATION
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SUMMARY OF WORK (200 words or less - underline keywords)

Clinical studies with the interferon inducer poly ICLC have been expanded. Clear beneficial effects have been seen in some diseases, specifically juvenile laryngopapilloma, multiple myeloma, and demyelinating neurological diseases. Toxicities are severe in very ill children with far advanced leukemia, consisting of fever, chills, hypotens myalgia; and modest in patients with a high performance index, consisting of a lesser degree of fever and myalgia.

PHS-6040
(Rev. 10-76)

Achievements The past year has been spent mostly in expanding the clinical investigations with poly ICLC 1) to determine whether there are areas where this drug holds promise and 2) what is the nature of the toxicities seen with the drug. In addition, we have spent some time in modifying the formulation and in trying to ameliorate some of the undesirable side effects. We have also made beginnings in developing a computer based storage system to facilitate retrieval of the large amount of data being developed.

1. Clinical investigations. A number of studies have been started; some have given encouraging results and some not.

a. Programs treating children with acute lymphatic leukemia are ongoing in collaboration with two groups: Drs. Krown and Oetgen at Sloan Kettering Institute and the Children's Cancer Study Group, a multi-institutional cooperative testing group. These studies have been done with very seriously ill children, with disease that had become resistant to all therapy. These children, in both programs, could not tolerate the levels of drug given in the Phase I test with Dr. Levine. Fever, hypotension and severe myalgia were the primary symptoms. At lower levels of drug, where toxicity was acceptable, only low levels of interferon were made. The Children's Cancer Study Group found enough evidence of effect on the disease that they are continuing to study alternate dosages and drug levels along with therapy for the fever. In preliminary studies with Dr. Stringfellow of Upjohn Company, we found evidence that the sera from these patients inhibits the production of interferon by tissue culture cells stimulated by interferon inducers. 2) That not all children are affected so adversely by poly ICLC is indicated from studies done with Dr. Levinthal of Johns Hopkins and Dr. Whisnant of the University of North Carolina. In these studies, 4 children with juvenile laryngopapilloma have been treated with the drug. Prior to treatment these young children had required surgery every week or two. When they are on the drug, the papilloma do not grow and surgery is not needed. They produce high levels of interferon 500-2000 u/ml of serum. So far, when they are removed from treatment, growth of the tumor re-ensues. These children have been able to accept levels of drug as high or even slightly higher than those that were found acceptable in the NCI study. There has been no hypotension, a modest transient granulocytopenia, no lymphopenia, a transient small rise in some liver enzymes, moderate fever and lethargy for 6-8 hours. Both investigators are eager to enlarge their studies. Of particular interest to NIAID is the probability that this is a disease of viral etiology. 3) Two studies are ongoing in multiple myeloma--the longest one is with Drs. Durie and Salmon at the University of Arizona. Five patients have been treated, all with beneficial effects. They have a reduction in myeloma proteins of $\geq$50%, bone marrow lesions have decreased in size, and a greatly improved feeling of health has been experienced. Myeloma patients present a special problem. Because of the fragility of the bones, they are discomfitted greatly if fever is allowed to develop to the point of shaking chills. So far, these patients have been given only moderate levels of drug, and have produced levels of interferon of 20-200 units/ml of serum. These patients show fever, only rarely is there leucopenia, occasionally there has been modest hypotension and there was one case of hypertension. Myalgia is common.

The other study of multiple myeloma is with Drs. Alexanian and Gutterman at M.D. Anderson. This study, as the one at Arizona, is with far advanced patients. All that can be said is that the patients are producing good levels of interferon. Toxicity is about the same as that seen by Dr. Durie. The group of M.D. Anderson has seen beneficial effects of exogenous interferon given to patients who were not too far advanced, but not in far advanced patients. 4) In studies with Drs. Engel and Salazar of the NINCDS, we have now looked at 8 patients with demyelinating neurological disease, 7 of the peripheral nerves and 1 of the central nervous system. All these patients were either paraplegic or had very severe loss of muscle control, to the point of not being able to walk or care for themselves. They were refractory to all standard therapy. All eight had moderate to marked improvement; 4 have been sent home for rehabilitation and job training. In these patients, toxicity has consisted of fever of 1-3 C, for 18 hrs., a reduction of lymphocytes to 20% of normal with subsequent return to normal, mild transient elevation of transaminases. There were no changes in blood pressure. These are diseases for which there is no effective therapy.

We have done some work to prepare poly ICL (without CMC) in an easier way than we have used, and have had some success. This complex produces about 1/3-1/4 as much interferon in monkeys than does poly ICLC. Toxicity studies should be done.

A contract has been let with Roswell Park Memorial Institute to study formulation and stability of poly ICLC.

A German Pharmaceutical company has applied for a license to manufacture poly ICLC as an immune adjuvant to be used with their vaccines. Other companies are considering so doing. Beginnings have been made to use the NIH computer to store and correlate the data being developed.

Relevance to NIAID and NIH:

Interferon inducers offer the potential of being broad spectrum antiviral and antitumor agents, a goal clearly within the mission of NIH. The immune potentiation possibilities of poly ICLC is directly in line with the mission of NIAID.

Future Plans:

High priority will be given to completing the program, now underway, to develop recording forms that all clinicians will use to record data about their patients. This data will be stored in the NIH computer under a program designed to retrieve information about toxicity, different lots of drug, beneficial effects, etc.

A few additional clinical programs are contemplated which will include life threatening illnesses associated with viruses.

Efforts will be made to reduce toxicity, including use of adjunct drugs, changes in scheduling and studying poly ICL (without CMC).

Publications

Levy, H.B.: Induction of interferon in vitro by polynucleotides and derivatives; and Preparation of derivatives. Interferons: Methods in Enzymology., S. Pestka, Ed., Academic Press, N. Y., N.Y.

Levy, H.B.: Induction of Interferon by polynucleotides in Interferon and Interferon inducers. Clinical Applications., Stringfellow, D.: Ed, Marcel Dekker, N. Y. 1980, pp. 167-186.

Levy, H. B., E. S. Stephen, D. Harrington, Dr. Engel, F. Riley and E. Lvosky. Polynucleotides in the Treatment of Disease. Augmenting Agents in Cancer Therapy, in press, Raven Press, N.Y., N.Y.

Levy, H. B., E. Lvovsky, F. Riley, D. Harrington, A. Anderson, J. Moe, I. Hilfenhaus, E. Stephen. Immune Modulating Effects of Poly ICLC., N. Y. Academy of Sciences Symposium on Interferon, in press.

Levine, A. S.; B. Durie, B. Lampkin; B. G. Leventhal and Levy, H. B. Interferon Induction, Toxicity and Clinical Efficacy of Poly (ICLC) in Hematologic Malignancies and Other Tumors. Second International Conference on Immunotherapy of Cancer: Present Status of Trials in Man. In press.

Levine, A. S., M. Sivulich, P. Wiernik; H. B. Levy. Initial Clinical Trials in Cancer Patients of Polyriboinosinic - Polyribocytidylic Acid Stabilized with Poly-L-lysine, in Carboxymethylcellulose (Poly(ICLC) a highly effective interferon inducer. Cancer Research 39, 1645-1650, May 1979.

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Champney, K. J., D. P. Levine, H.B. Levy, A. M. Lerner. Modified polyriboinosinic-polyribocytidylic acid complex: sustained interferonemia and its physiological associates in humans. Infection and Immunity (831-837) Sept. 1979.

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Krown, S. E.; K. Keir, W. Stewart, M. S. Pollack, S. Cunningham, Rundle, Y. Hirshant, C. M. Prisley, H. B. Levy and W. J. Oettgen. Phase I trial of poly ICLC in patients with advanced cancer. M. Augmenting agents in cancer therapy, Raven Press, m. Chirigos, Ed., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-A1-00190-02 OSD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Molecular Genetics of Eukaryotic Cells and Their Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	Roy Repaske, Biochemist	OSD, NIAID
	Dean Hamer, Staff Associate	OSD, NIAID
	Mark Israel, Research Associate	OSD, NIAID
	Yoshiaki Ito, Visiting Scientist	OSD, NIAID
	Arifa Khan, Visiting Fellow	OSD, NIAID
	Kamal Chowdhury, Visiting Fellow	OSD, NIAID
	George Pavlakis, Visiting Fellow	OSD, NIAID
	Susan Light, Research Associate	LVD, NIAID

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COOPERATING UNITS (if any) Wallace P. Rowe, LVD, NIAID; Edward Scolnick, LTVG, NCI; Dr. Claude Garon, LBV, NIAID; Dr. John Gerin, Division of Molecular Virology & Immunology, Georgetown Univ.School of Med.&Dentistry; Dr. Peter Howley, Laboratory of Pathology, NCI; Dr. Fusao Hirata, Lab. of Clin. Sci., NIMH; Dr. Eiichi Soeda,*

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SUMMARY OF WORK (200 words or less - underline keywords)

The principal goal of this unit continues to be the biochemical characterization of eukaryotic and animal viral genes. Attention has been focussed on the segments of papovavirus and murine leukemia virus genomes responsible for the establishment and maintenance of the transformed state. In this regard, the portions of polyoma virus DNA mediating tumorigenesis has been delineated and the structure and intracellular location of the protein kinase activity of viral middle T antigen has been investigated. A subgenomic segment of AKR ecotropic murine leukemia provirus, that specifically reacts with the envelope region of endogenous ecotropic retroviruses, has been cloned in E. coli and used as a probe to identify such sequences in normal mammalian DNAs. SV40 DNA recombinants containing mouse and human globin genes and the human growth hormone gene have been constructed and the gene products produced in monkey kidney cells evaluated. Risk assessment experiments involving EK1 derivatives of E.coli K12 containing oligomers of polyoma DNA, Harvey sarcoma virus and dilysogens of polyoma DNA were initiated.

Project Description

The research activities of the DNA Recombinant Research Unit involve investigations of the regulation and structural organization of eukaryotic cellular and animal viral genes. Our group has had a long standing interest in tumor viruses focussing on the structure and expression of integrated viral genomes. One of these tumor viruses (SV40) is being used as a vector for the propagation and functional analyses of eukaryotic genes previously cloned in E.coli K12. Experiments evaluating potential risks associated with recombinant DNA experimentation have been carried out in the containment facility located at the Frederick Cancer Research Center and P2 laboratories in Bethesda.

During the year, Dr. Hardy Chan resigned to take a position at Syntex Corporation, Palo Alto, California. Drs. Arifa Khan and George Pavlakis joined the group as Fogarty Visiting Fellows working with Drs.Martin and Hamer, respectively. Dr. Susan Light, Research Associate, LVD, NIAID, began working with Dr. Ito in January 1980.

Research Accomplishments

I. The Oncogenic Potential of Papovaviruses

A. Viral Genes Mediating Transformation (Israel and Chowdhury)

Previous studies carried out in this laboratory demonstrated that polyoma virus (PY) large tumor (T) antigen (Ag) and viral gene sequences which specifically encode large T Ag were absent in PY induced tumors. This result indicated that PY large T Ag was not required for the maintenance of the transformed state. To define more precisely which portions of the viral genome were absolutely required for the initiation of transformation or tumorigenesis, cloned segments of PY DNA were inoculated into newborn hamsters or used to transform a continuous rat cell line. Cloned viral DNA representing approximately one-half of the early gene region and postulated to encode PY middle and small T Ags induced tumors in hamsters and transformed cells in culture. Transformed or tumor cell lines resulting from this experiment contained viral middle and small T Ags. Furthermore, hamster tumor cell lines containing these two antigens possessed a fully transformed phenotype and contained the PY tumor specific transplantation Ag but no large T Ag. A cloned PY DNA segment in which the DNA sequence specifying the carboxyterminal portion of middle T Ag had been deleted failed to induce tumors or transform cells in culture. These results indicate that about 25% of the viral genome is sufficient for the expression of the oncogenic activity of PY and provides further evidence that a functional middle T Ag plays a key role in this process.

During the past year copies of PY DNA which are integrated into the chromosome of tumor cell lines have been cloned using a lambda-phage - E. coli K12 host vector system. The clones obtained are currently being examined by restriction enzyme digestion, electron microscopy, and DNA sequencing techniques to more fully evalute the organization and expression of the viral genome in tumor cell lines.

B. Viral Encoded Proteins and Transformation (Ito and Light)

Since the initial detection of PY middle T Ag in the plasma membrane of virus transformed cells by Ito and his colleagues and its demonstrated association with a protein kinase activity in such cells, considerable effort has been directed toward determining its chemical characterization, intracellular localization and functional activity. Our earlier studies had suggested that PY middle T Ag was situated on the inner surface of the plasma membrane. Experiments carried out during the past year employing two types of plasma membrane vesicles ("outside-out" and "inside-out"), however, demonstrated that the phosphorylation activity associated with PY middle T Ag is localized on the outer surface of the plasma membrane. In view of the overwhelming evidence that middle T Ag was essential for oncogenesis, its location on the outer plasma membrane raised the possibility that tumor growth might be inhibited by blocking the surface phosplorylation of this viral encoded polypeptide. Experiments have, therefore, been initiated with sera obtained from animals with regressing tumors, provided by Dr. George Klein, Karolinska Institute, Sweden, to ascertain if the phosphorylation of middle T Ag can be inhibited.

The transforming activity of a PY deletion mutant which synthesizes a non-functional middle T Ag was studied in collaboration with Dr. Roland Sief, NIAMDD. Transformed cells appearing after infection with the mutant virus were phenotypically similar to SV40 (which cannot synthesize a middle T Ag) transformants suggesting that the oncogenic activity of PY may also be mediated by other early viral gene products. In another group of experiments, the PY large T Ag induced under various conditions was examined by tryptic peptide fingerprint mapping. The results from these experiments suggests that there is a modification of PY large T Ag which appears to be required for its ability to initiate viral DNA replication and that the few PY transformed cell lines which have been shown to synthesize large T Ag may actually contain a related but non-functional viral encoded large T Ag.

II. The Molecular Structure of Murine Retroviruses (Chan, Khan, Repaske, Martin)

These studies represent a collaborative effort between the DNA Recombinant Research Unit and the Laboratory of Viral Diseases, NIAID with the goal of characterizing endogenous murine leukemia viruses (MuLVs). During the past year, we have prepared recombinant plasmids containing different segments of the ecotropic MuLV provirus of AKR mice (AKV), and presently have an ensemble of DNA probes which hybridize to specific portions of the virus genome. We have constructed a DNA probe from one of the recombinant plasmids encoding a portion of the viral envelope of AKV that specifically hybridizes to endogenous ecotropic DNA sequences present in normal mouse DNA and which exhibits no reactivity to xenotropic or MCF proviruses or to the DNA of NFS mice, a strain which does not express ecotropic viruses. Annealing of the labeled ecotropic specific DNA probe to AKR or BALB/c mouse liver DNAs revealed the presence of 3 and 2 copies of endogenous ecotropic proviruses, respectively. An unexpected result was the reactivity of the ecotropic specific probe with DNAs prepared from NZB, C57L, and 129 mice, strains from which ecotropic MuLV have not been recovered. The size and quantities of endogenous ecotropic proviral DNA detected in these three strains of mice is compatible with the presence of multiple subgenomic viral DNA segments that may exist as tandem arrays integrated into mouse chromosomal DMA.

Using the recombinant plasmid DNA preparations described above which span different segments of AKV provial DNA, we examined the tissues of other mammalian species for the presence of related endogenous proviruses. As expected, MuLV-like sequences were readily detected in tissues of other rodents such as rat and hamster. Employing non-stringent nucleic acid hybridization techniques and subgenomic segments of cloned AKV DNA, we were able to detect the presence of several copies of retroviral sequences in African green monkey liver DNA preparations . Sequences encoding the reverse transcriptase and a portion of the glycoprotein envelope of AKV were detected in normal monkey DNA; DNA probes specifying the capsid protein or the long terminal repeat of the AKV proviruses reacted weakly,if at all, with monkey liver DNA. On the basis of this result, we recently cloned an endogenous proviral DNA segment from monkey cells and are in the process of characterizing it biochemically. It is anticipated that this endogenous monkey proviral DNA can be used as a hybridization probe to identify and eventually, to clone, related DNA segments from human DNA.

It has been previously shown that all proviruses are flanked by a 600-700 base pair DNA segment, the long terminal repeat (LTR), which is located at the 3' and 5' termini of integrated rotroviral DNA. In collaboration with Dr. E. Soeda, National Institute of Genetics, Mishima, Japan, we have determined the nucleotide sequence of the 650 base pair LTR present in cloned Harvey sarcoma DNA. The sequence determined is quite similar to an analogous segment of Moloney sarcoma virus recently sequenced by another laboratory. Comparison of the two DNA segments of these related murine sarcoma viruses indicates considerable conservation of sequences which specify regulatory functions (e. g. polyadenylation and splicing sites) and dissimilarities of sequences located in "silent" regions of the terminal repeat. The sequence of the LTR of a cloned ecotropic MuLV has been partially determined and found to contain a long stretch of inverted sequences. The structure of the LTR of the ecotropic MuLV when obtained will be compared to the LTR present in xenotropic and MCF MuLVs.

III. Regulation of Eukaryotic Genes (Hamer and Pavlakis)

Using SV40 as a vector to introduce new genetic information into cultured animal cells, the expression of several different eukaryotic genes in African green monkey kidney cells was studied. The SV40 recombinants which have been constructed and evaluated during the past year contain: 1) a mouse globin gene and several deletion mutants derived from it; 2) a human α thalassemia gene; 3) a human growth hormone gene; 4) xenopus 5S and tRNA genes; 5) the promoter for the chicken ovalbumin gene; and 6) a portion of the hepatitis virus type B genome.

The mouse α-globin gene promoter is efficiently and accurately recognized in monkey kidney cells infected with SV40-globin recombinants. The results obtained indicate that kidney cells contain all of the factors essential for α globin gene transcription and can splice, polyadenylate, and translate mouse α globin mRNA. Companion experiments carried out with deletion and substitution mutants of the mouse α globin gene which we constructed indicated that nucleotide sequences 18 to 309 base pairs 5' to the initiation site are required for accurate transcription of globin mRNA.

Cultured monkey cells infected with SV40-human growth hormone gene recombinants actively synthesize growth hormone. The hormone is appropriately processed and can be detected intracellularly as well as in the tissue culture medium. Careful examination of the medium revealed that human growth hormone constituted the major protein secreted by the monkey kidney cells. These experiments are the first example of the biosynthesis of an authentic hormone through recombinant DNA technology and represent a potential source of homogeneous human growth hormone.

IV. Assessment of Risks Associated with Recombinant DNA Research
(Drs. Martin and Rowe)

We have extended our previous studies which have evaluated the potential biological activity of *E. coli* derivatives containing animal viral DNA segments. Experiments published by us in 1979 indicated that EK2 derivatives of *E. coli* K12 containing a potentially infectious polyoma DNA recombinant were neither tumorgenic in newborn hamsters nor capable of inducing a virus infection in weanling mice. We are presently conducting similar experiments with EK-1 derivatives of *E. coli* K12 and have inoculated animals with bacteria harboring a recombinant plasmid containing a head-to-tail dimer of polyoma virus DNA or with lambda lysogens. Similar experiments are also planned utilizing wild type *E. coli* as well as feeding studies of EK-1 derivatives to germ-free animals to ascertain the long-term effect of potentially infectious viral clones in the intestinal tract of animals.

E. coli K12 containing Harvey sarcoma virus DNA was recently inoculated into mice and hamsters. Preliminary results of this experiment show that neither purified recombinant DNA preparations nor bacteria containing such DNA induced tumors during a 5 month observation period.

Publications

Chan, H. W., Garon, C. F., Chang, E. H., Lowy, D. R., Hager, G. L., Scolnick, E. M., Repaske, R., and Martin, M. A.: Molecular cloning of the Harvey sarcoma virus circular DNA intermediates. II. Further structural analyses. *J. Virol. 33*: 845-855, 1980.

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Moore, J. L., Chowdhury, K., Martin, M. A., and Israel, M. A. Polyoma large tumor antigen is not required for tumorigenesis mediated by viral DNA. Proc. Nat. Acad. Sci. 77: 1336-1340, 1980.

Honors

Dr. Martin continues to serve on the Editorial Board of the Journal of Biological Chemistry. He was invited to give seminars at Columbia, Johns Hopkins, Rockefeller Universities and the University of California Medical School, San Francisco. He chaired a session and delivered a paper at the Gordon Conference on Eukaryotic Cells in July 1980. He is a member of the NIAID Risk Assessment Working Group and was invited to attend the Recombinant DNA Risk Assessment Workshop held at Pasadena, California in April 1980.

Dr. Hamer was invited to give seminars at the University of Virginia, Rutgers, University of Colorado, George Washington, Duke and Yale Medical Schools and the Massachusetts Institute of Technology. He was also invited to the Cell Biology Meeting, Toronto, Canada; Eukaryotic Genome Meeting, Seliac, France; Parasitology and Recombinant DNA Workshop, Woods Hole, Massachusetts and three Gordon Conferences. Dr. Hamer also taught courses at Cold Spring Harbor, Woods Hole Marine Biological Laboratory and at the Given Institute of Pathobiology, Aspen, Colorado.

Dr. Yoshiaki Ito was an invited speaker in the Symposium on Tumor Immunology at the Fourth International Congress of Immunology held in Paris, France, in July 1980. He was also invited to give seminars at McGill University and at Montreal and Sherbrook Medical Schools, Canada.

LABORATORY OF BIOLOGY OF VIRUSES
1980 Annual Report
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Annual Report of the Laboratory of Biology of Viruses
National Institute of Allergy and Infectious Diseases, NIH
October 1, 1979 to September 30, 1980

The goal of the Laboratory of Biology of Viruses is to determine how viruses replicate. At the molecular level, this involves investigation of the structure of the virion and of its purified component parts, of mechanisms of replication and transcription and expression of the viral genome. Emphasis is also placed on cell-virus interactions including the process of cell transformation. We believe that information of this kind, although difficult to obtain, is fundamental to understanding disease processes and vital to achieving a rational basis for chemotherapy.

Papovaviruses

Transcription and Replication of SV40

During viral infection, the virus enters the cell, and in the case of SV40, the DNA viral genome is transported to the nucleus. It is not clear what fraction of the protein which is present in virions remains associated with the viral DNA within the cell. Our current understanding of the infectious cycle indicates that transcription of the viral DNA is one of the earliest events, and that the viral DNA which is used as the template is associated with proteins. The precise nature of the nucleoprotein complex which is the functional transcription template is not defined. The enzyme which synthesizes this early viral RNA is the cellular RNA polymerase II. This is the enzyme which also synthesizes cell messenger RNA. We have continued our studies to define the mechanism of transcription and to understand how the synthesis of different populations of RNA are regulated during the infectious cycle.

Simian Virus 40 (SV40) provides an excellent model system for investigating the process of transcriptional regulation in eukaryotic cells, since some properties of viral transcriptional complexes and mRNA molecules have been determined. In addition, the entire nucleotide base sequence of SV40 DNA has been determined and like most eukaryotic chromatin, during the SV40 infection cycle, cellular histones H_2A, H_2, H_3, and H_4 are bound to the viral DNA.

Localization of RNA Polymerase Promoters on SV40 DNA

Transcription of SV40 DNA by the Escherichia coli RNA polymerase has been investigated as a model system for the development of methods applicable to the study of the mechanisms of transcription by the mammalian RNA polymerase II. E. coli RNA polymerase binding sites have been previously investigated by electron microscopy. Except for one binding site, their location on the SV40 DNA map is only approximate.

While E. coli RNA polymerase recognizes specific promoter sites, it does not recognize specific termination sites on SV40 DNA, and so RNA molecules that are formed are not of a defined size. We have developed a method that allows for the production of discrete RNA species. Preformed RNA polymerase-SV40 DNA transcriptional complexes are cleaved with a "single-cut" restriction endonuclease (Bam HI, Eco RI). These cleavages generate termination sites at precisely defined locations. The "run-off" RNA subsequently synthesized on these linearized complexes can be resolved into discrete bands by gel electrophoresis. Fifteen major initiation sites have been identified using this method. The determination of the size of each of these RNA species and of the direction of their transcription has enabled us to construct a detailed map of the promoters recognized by the E. coli RNA polymerase on SV40 DNA.

The determination of the sequence of the 5' ends of some of the transcripts initiated is presently being carried out and the precise location of these promoters will allow us to compare the DNA sequences upstream from these sites and provide a better understanding of the nature of the interactions between the RNA polymerase and the DNA.

Studies with eucaryotic RNA polymerase II

The studies with E. coli RNA polymerase have served as an initial guide in our studies with mammalian RNA polymerase II. Highly purified preparations of RNA polymerase II, isolated from calf thymus, are being utilized to transcribe linear, relaxed (nicked) and superhelical SV40 DNA in in vitro transcription systems. With each of these templates, α-32P-UTP is incorporated into discrete products. However, the ^{32}P label products produced with linear and relaxed (nicked) DNA are not "classical transcripts"; instead these products appear to be covalent aducts of RNA and DNA. At present the possible significance of these aducts is being investigated. In addition, RNA products produced from superhelical DNA are being characterized with regard to initiation and termination sites. Once the interaction of RNA polymerase II and DNA have been worked out in this relatively simple system, it will be possible to test the effect of various cellular factors on the specificity of transcription.

Transcriptional Properties of SV40 Nucleoprotein Cores

During the past year, we have been characterizing what appears to be a "chromatin activation" process that occurs during SV40 virion maturation. Two distinct SV40 virus particles are produced during a lytic infection cycle. The initial viral particle (immature) is found only in the infected cell nuclei and is a precursor to the mature viral particle, which is released from the infected cell. Analysis of the two virion populations indicate that both have a sedimentation value of 240S, a density of 1.33 g/cc and are sensitive to in vitro EGTA-DTT dissociation, indicating similar capsid structure. However, when the viral chromatin packaged inside the virion is examined, significant differences appear. The DNA-protein complex of the immature virus particle has a sedimentation value of 75S and contains cellular histones H_1, H_2A, H_2B, H_3 and H_4. Micrococcal nuclease digestion of immature virion chromatin gives a typical nucleosomal DNA ladder pattern indicative of higher order chromatin structure. The rate of incorporation of ribonucleoside triphosphates into RNA with the immature chromatin particle is 5% of that obtained with SV40 form 1 DNA.

The 110S chromatin particle (SV40 core) packaged inside the mature SV40 virion contains acetylated histones H_2A, H_2B, H_3, H_4. No H_1 can be detected in the mature virion. In place of H_1, viral proteins VP_1 and VP_2 are found associated with the chromatin particle. Micrococcal nuclease digestion of mature virion chromatin results in only submonomer nucleosomal DNA bands, indicative of a loose association of nucleosomes with the viral DNA. Transcriptional studies performed with SV40 cores indicate that they are potentially active transcriptional complexes. The transcriptional activity of these complexes is extremely high and is similar to the activity obtained with purified SV40 F1 DNA; 20 fold higher than the rate observed with immature virion chromatin. Control experiments demonstrate that only when VP_1 and VP_2 are associated with the mature virion core is the transcriptional rate high; i.e. removal of VP_1/VP_2 to generate a DNA-histone (minichromosome) complex results in a decreased transcriptional efficiency. These results suggest that the viral proteins play an important role in the "activation" of the nucleohistone complex.

The SV40 chromatin modifications which appear to occur during viral maturation, i.e. histone acetylation, loss of histone H_1 increased sensitivity to DNAse 1, and association of non-histone proteins are all characteristic of the transition from transcriptionally inactive to active chromatin in eukaryotic cells. Further analysis of the chromatin transition and structure of the mature SV40 nucleoprotein core should provide an excellent system for analysis of eukaryotic RNA transcription.

Site Specific Mutagenesis

The purpose of the project is to define sites at the nucleotide level that control DNA replication and transcription of SV40. Purified DNA from SV40 has been nicked and gapped at a site known to contain signals for the two previously mentioned functions. This DNA served as a substrate for chemical mutagenesis that converts cytidine to uridine specifically in single-stranded nucleic acids. The chemically modified DNA has been inserted into a bacterial plasmid for the isolation and amplification of individual molecules. Specific chemical alterations in the DNA will be correlated to an altered function in the life cycle of SV40.

Parvoviruses

Studies of Transcription

In our studies of the in vivo transcription of KRV, we have utilized nuclei isolated from rat nephroma cells infected with KRV. We have isolated viral specific transcripts from the infected cells as early as 8 h after infection. The predominant viral RNA species is 19-22S and represents a transcript of 60-75% of the viral single-stranded DNA. A viral RNA transcript representing 95-100% of the viral genome can also be detected. On the basis of α-amanitin inhibition studies, synthesis of the KRV transcript is believed to be mediated primarily by cellular RNA polymerase II. Over 65% of the viral-specific RNA in the isolated nuclei is polyadenylylated on the 3' terminus. The 5' terminus of the RNA is capped in vitro by the sequence $m^7G(5')ppp(5')A$. Incorporation of β - ^{32}P ATP into the 5' cap sequence suggests that initiation of viral RNA synthesis occurs in the infected isolated nuclei. We hope to study further in vitro synthesis of viral specific RNA by lysing the nuclei and utilizing this cell free system to determine the components required for viral transcription.

In vitro studies of RNA transcription have complemented the in vivo studies and extended our knowledge of viral transcription. We have isolated from the polysomes of infected cells two major viral specific RNAs sedimenting in sucrose gradients at 21S and 12S. The 21S RNA has a molecular weight of approximately 1.05×10^6 and would represent a transcript of 65% of the genome. The smaller 12S RNA has a molecular weight of 0.3×10^6 and would represent a transcript of 20% of the genome. Both of these DNAs are transcribed from the viral strand of the viral DNA. No functional viral RNAs have been found which hybridize with the complementary strand of the viral DNA. Two techniques have now been used to map these mRNAs to the viral genome. We have mapped the fragments of several restriction enzymes to the KRV viral genome. Using the mapped restriction fragments, the major cytoplasmic viral RNA (21 S) has been mapped to the viral genome using the "Southern" blotting technique. This has been complemented by electron microscopy of "R loops" or RNA-DNA duplexes. Both techniques indicate that the 21 S viral mRNA maps from approximately 0.38 to 0.98 on the viral DNA strand. Looking at total cellular RNA we can find viral specific transcripts the full length of the viral genome. We hope to study this transcript further to determine posttranscriptional processing occurs by simple cleavage or splicing and what regions are not transcribed into mRNA.

Adenovirus-Associated Viruses

The main objectives in studying defective human parvoviruses (AAV) are to define specific biochemical mechanisms used in synthesizing their DNA, RNA and proteins, to identify and characterize the helper virus-mediated step(s) required for their replication, to relate biochemical findings to normal cellular processes, and to determine conditions for selective interference of both AAV and Helper virus (adenoviruses, herpesviruses) infection.

Mechanism of AAV DNA Synthesis

AAV DNA replicative intermediates have been synthesized in soluble extracts prepared from infected cell nuclei, and conditions that yield de novo initiations of DNA synthesis, as well as elongation of molecules already initiated in vivo, have been defined. Analysis of the kinetics of DNA synthesis and of structural features of completed products further support our initially reported model for the self-primed replication of AAV DNA. Further refinement of this system is in progress so that most, if not all, factors required for synthesis can be characterized. An ultimate goal is to determine conditions needed for repeated reinitiations of DNA synthesis *in vitro*.

Adenovirus Genes Required for Replication of AAV

AAV multiplication is dependent upon the expression of one or more early adenovirus genes. In current studies, DNA transfection experiments have been utilized to map the required adenovirus genes. Furthermore, functions provided by these genes have been determined by analysis of AAV macromolecular synthesis following transfection with selected adenovirus DNA fragments. In most instances, it is likely that these genes play similar roles in the replication of the helper adenovirus. Clearly, AAV provides a useful marker for analyzing specific functions of early, regulatory adenovirus genes. It is at this stage of the replication cycle that virus growth may be most vulnerable to selective interference.

Structure-Function Relationships of DNA Binding Protein

The Adenovirus 72K DNA-binding protein is synthesized during the early period of adenovirus infection and appears to be a multifunctional element. It is required for viral DNA synthesis (initiation and elongation) as well as for efficient expression of several late viral proteins. It also may be required for synthesis of all AAV structural polypeptides. Our present studies suggest that certain structural variations in the 72K protein can be related to the loss or gain of specific functions mediated by this protein. Investigations will continue with the objectives of determining the exact nature of such structural variants, their primary sites, and how and where these specific structures interact to carry out their regulatory functions.

Adenoviruses

Structure of the Viral Genome

Our initial work on the inverted terminal repetition found in the DNA of several human adenovirus serotypes also contained the observation that the length of these terminal sequences differed among the major serotype groups. A possible association between the increased length of the redundant segment and increased oncogenic capability was suggested. Therefore, we were interested in mapping the terminal nucleotide sequence of "highly oncogenic" adenovirus serotype 18 (Ad 18) and comparing this map with members of other major groups.

The structure of the DNA isolated from plaque-purified prototype Ad 18 has been analyzed by cleavage with restriction endonucleases, by electron microscope heteroduplex techniques and by nucleotide sequence analysis. A physical ordering of fragments was obtained by reciprocal digestion of isolated fragments and heteroduplex mapping. End fragments were identified by their susceptibility to exonuclease digestion and by the presence of covalently attached terminal proteins. Based on nucleotide sequence data, the inverted terminal repetition of Ad 18 is 165 nucleotides long and appears to be identical in sequence at both termini. The inverted terminal repeat in members of group B (Ad 3, 7) is reported to be 136 nucleotides in length while members of group C (Ad 2, 5) are 103 nucleotides long. Adenovirus serotype 12, also a member of the highly oncogenic group A, has a 162 nucleotide inverted terminal repeat that is not completely homologous to Ad 18. A plaque-purified varient of Ad 18 was isolated whose DNA measured approximately 4% longer in total length than the prototype strain. A structural comparison of the DNA of this isolate with that of the prototype strain revealed a restriction endonuclease cleavage pattern which appeared to differ only in the size of the terminal fragments. This finding was confirmed by electron microscope heteroduplex comparisons. Furthermore, the inverted terminal repetition of this isolate, which represents 3-4% of total genome length (or about 1300 nucleotides), did not replace any sequences that adjoin the inverted repeat of the prototype genome, nor did it contain any apparent internal molecular rearrangement. It is not known how these terminal repetitions originate, but it is apparent, however, that the mechanism involved can produce a variable but accurate inverted repeat. Since both the origin of replication and transforming activity have been mapped near the end of the genome, these isolates provide valuable reagents for the more detailed study of viral replication or carcinogenesis, or both.

Pox Viruses

Our primary objective is to determine the molecular events that lead to the selective transcription of DNA and subsequent processing of viral and eukaryotic messenger RNA (mRNA). Poxviruses provide a unique and important system for such studies since the enzymes needed for transcription and mRNA modification are packaged within the core of infectious virus particles. During the past year, several important advances have been made. For the first time, the RNA polymerase has been solubilized and purified from vaccinia virus particles. DNA recombinant technology has been applied to vaccinia virus and genome segments have been cloned in phage and plasmids. This has allowed us to determine the presence of 30 tandem repetitions of a 70 bp sequence, present near each end of the genome, which are presumably used for DNA replication. Several early mRNAs and polypeptides have been mapped within the long 10,000 bp inverted terminal repetition. Significantly, no evidence of RNA splicing has been found. Continued studies on the enzymatic mechanism of capping mRNA has led to the purification of a cap specific mRNA (nucleoside-2-)-methyltransferase from HeLa cells and capping enzyme from wheat germ. In addition, a transfer RNA (cytosine-5-)-methyltransferase was purified from HeLa cells and shown to specifically methylate cytidine residues within the transition sequence between the extra arm and the righthand loop of tRNA.

I. Post-transcriptional modification of eukaryotic RNA. Members of this research group previously identified the 5' terminal cap structure $m^7G(5')pppN$, consisting of a 7-methylguanosine (m^7G) linked by a 5' to 5' triphosphate bridge to one or two consecutive 2'-O-methylated ribonucleosides (N^m) present in viral and cellular mRNAs. The mechanism of cap formation was determined by purifying and characterizing the responsible enzymes from vaccinia virus. Subsequently, by isolating enzymes from HeLa cells, we demonstrated that cap formation of eukaryotic and viral mRNAs occur by similar, although slightly different, mechanisms. During the past year, we have extended this work by purifying a cap specific RNA (nucleoside 2'-)-methyltransferase from HeLa cells and an RNA guanylyltransferase (capping enzyme) from wheat germ. A transfer RNA (cytosine-5-)-methyltransferase was purified from HeLa cells and its substrate specificity was determined.

A. Purification and characterization of HeLa cell RNA (nucleoside-2'-) methyltransferase. A specific assay was developed for cap-specific RNA (nucleoside-2'-)-methyltransferase. The substrate for this assay was prepared by using purified vaccinia virus capping enzyme, synthetic poly(A, $[\alpha\text{-}^{32}P]$GTP and S-adenosylmethionine (AdoMet). The substrate, $m^7G(5')p\overset{*}{p}A(pA)$, and AdoMet were then incubated with enzyme fractions and the formation of $m^7G(5')\overset{*}{p}pA^m(pA)_n$ was detected by RNase T_2 digestion and high voltage DEAE paper electrophoresis. Using this assay, a specific RNA (nucleoside-2'-) methyltransferase was purified. Characterization of the enzyme is now in progress.

B. Purification of capping enzyme from wheat germ. Capping enzymes are extremely useful for specifically labeling the ends of RNA molecules. After receiving numerous requests for vaccinia virus and HeLa cell capping enzymes, which are difficult to purify in large quantities, we decided to attempt a purification from a more available source. Accordingly, wheat germ was chosen. Thus far, we have achieved a nearly 2,000 fold purification of the enzyme and have made a preliminary characterization of its catalytic activities which are more similar to the HeLa cell capping enzyme than to the vaccinia virus enzyme. That is, it has no associated RNA triphosphatase activity and requires a diphosphate terminated polyribonucleitide. Also, like the HeLa cell enzyme, it has no associated RNA(guanine-7-)methyltransferase.

C. Post-transcriptional modification of transfer RNA: purification and characterization of HeLa cell tRNA(cytosine-5-)-methyltransferase. Eukaryotic cells contain a large number of different tRNAs that have similar nucleotide modifications such as methylation. To better understand protein nucleic acid interactions, we have purified a tRNA (cytosine-5-)-methyltransferase from HeLa cells. The enzyme specifically methylates cytidine residues within the transition sequence between the extra arm and the righthand loop of tRNA. The ability of the enzyme to methylate different tRNAs in the same part of the molecule suggests that the enzyme recognizes a general or limited sequence. Since RNase T_1 oligonucleotides of tRNA were not substrates, appropriate secondary or tertiary structure is probably needed in addition to the proper general sequence. Such a feature would be advantageous in allowing a single enzyme to methylate a large number of different tRNAs.

II. Organization and transcription of the vaccinia virus genome. Vaccinia virus, a member of the poxvirus family, provides a unique system for studying the synthesis and processing of mRNA from a DNA genome; all enzymes necessary for the synthesis of functional mRNA are contained within the infectious virus particle. The goal of this project is to define the roles of all enzymes and protein factors involved in the regulation of transcription. During the past year, we have used DNA recombinant methods to clone portions of the vaccinia virus genome and to determine the organization of some early transcriptional units. Another major achievement has been the purification of the vaccinia RNA polymerase.

A. Tandem Repeats within the Inverted Terminal Repetition of Vaccinia Virus DNA. Last year, we demonstrated that the same 10,000 base pair (bp) sequence is present at both ends of the vaccinia virus genome. To facilitate further studies, this segment of the genome was cloned in coliphage lambda. This permitted more detailed analysis which led us to detect the presence of direct tandem repetitions of a 70 bp sequence. These tandem repetitions begin about 200 bp from the very end of the genome. There are 13 tandem repetitions, followed by a 435 bp unique sequence, followed by 17 tandem repetitions of the same 70 bp sequence. Thus, there are 30 repetitions of this sequence at each end of the genome. These sequences are not transcribed and we have proposed that they function to accelerate cyclization of single strands of DNA during replication.

B. Analysis and mapping of mRNA and polypeptides encoded within the inverted terminal repetition. Early vaccinia polypeptides encoded within the terminal repetition were mapped by cell-free translation of mRNA that was selected by hybridization to restriction fragments and separated strands of a recombinant lambda phage. The results, which were confirmed by hybrid arrest of translation, indicated that polypeptides of 7,500 (7.5K) 19,000 (19K) and 42,000 (42K) daltons map at approximately 3.2 to 4.3, 6.5 to 7.2 and 7.2 to 8.3 kbp from the end of the genome, respectively. mRNAs for the 42K and 7.5K polypeptides are transcribed towards the end of the genome whereas mRNA for the 19K polypeptide is transcribed in the opposite direction. The mRNAs coding for these polypeptides were resolved by agarose gel electrophoresis. No evidence for interrupted genes was obtained by electron microscopy or nuclease S1 analysis of RNA·DNA hybrids. Thus, RNA splicing, which has been demonstrated for DNA viruses that replicate within the nucleus of infected cells, does not appear to be involved in synthesis of these first vaccinia virus mRNAs to be examined.

C. Purification of the vaccinia virus RNA polymerase. The DNA-dependent RNA polymerase was extracted from vaccinia virions and purified to near homogeneity. The native enzyme has a molecular weight of approximately 500,000 and can be dissociated into polypeptides of 140,000, 137,000 37,000, 35,000, 31,000, 22,000 and 17,000 daltons. Activity is dependent on a single-stranded DNA template, all 4 ribonucleoside triphosphates, and Mn^{2+}. The vaccinia virus RNA polymerase can be differentiated from prokaryotic and eukaryotic RNA polymerases by its resistance to rifampicin and α-amanitin, respectively.

Structural Studies of the Vaccinia Genome

Restriction enzyme digests of vaccinia virus DNA usually produce 15 or more unique segments which are separated by agarose gel electrophoresis. The large number of pieces makes it difficult to order the segments on a physical map. The restriction enzyme BglI, however, cleaves vaccinia DNA into only four large pieces A, B, C and D, with masses of 72, 28, 14 and 7 X 10^6 daltons. These segments can be separated by sucrose gradient centrifugation and they can be recovered in 100 mg quantities with less than 20% cross contamination from a single sedimentation run. Each of these four segments is digested with a second restriction enzyme, such as XhoI, and the electrophoretic pattern of the segments formed by the double digest is compared with the pattern obtained by digestion of intact DNA with Xho alone. This procedure identifies those Xho segments which are contained intact in each Bgl segment. These latter segments are large so they are usually cleaved at several sites by a second restriction enzyme. Since the order of BglI segments has been determined as C, D, B, A, analysis of the double digest gives an approximate location of many of the Xho segments. BglI digests can be treated with SmaI which cleaves only the A segment into two pieces of 48 and 24 X 10^6 daltons. These two enzymes divide the vaccinia genome into five large pieces into which all other segments can be grouped. Using these two enzymes and additional techniques, physical maps of SalI, XhoI, HindIII and BglI have been completed and a map for KpnI has almost been finished.

BglI cleavage leaves a protruding 3' end. Duplex DNA has been labeled at this site with either $dNTP^{32}$ or $rNTP^{32}$ by using terminal transferase in the presence of Co^{++}. Restriction segments with several labeled nucleotides at one end have been used for mapping by a partial digestion method. BglI segments can be sequenced by labeling with CTP^{32} or GTP^{32} and then removing all but the terminal P^{32} nucleotide with ribonuclease.

Since sequence analysis is facilitated by having larger quantities of DNA available, some of the smaller segments of vaccinia virus DNA are being cloned in an E. coli plasmid system. The plasmids are pBR327, a deletion mutant of pBR322, and pBR328, a deletion mutant of pBR325. SalI segment H has been cloned in pBR328 and we are currently trying to clone all or part of SalI segments C, D and E and XhoI segments C and D in pBR328, since these segments are hard to separate by agarose gel electrophoresis. Both Sal segments C and H have BglI sites.

HeLa cells have been labeled with 30 min pulses of ^{3}H-thymidine at different times after infection with high multiplicities of vaccinia virus to try to locate the origin of viral DNA replication. With these conditions, viral DNA synthesis peaks at 2.5 hours after infection. From 2 to 3.5 hours post infection, alkaline and neutral sucrose gradient sedimentation runs show labeled molecules of different size with less than 10% of the DNA in full length molecules. Electrophoresis of HindIII digests of these rapidly labeled full length molecules isolated from the cytoplasm shows a distribution of counts which is similar to the normal pattern. There are two additonal peaks, one located between segments F and G and the other between segments I and J. Also, the end segments B and C are underlabeled which is consistent with a region where replication begins. HindIII digests of full length DNA isolated from nuclei of cells infected 2.5 hours earlier also gives a distribution of label similar to the usual pattern except that peaks A ad B are underlabeled and the background level of counts is higher than that found for the cytoplasmic DNA. The labeled viral DNA isolated from the nucleus is clearly contaminated with cellular DNA and this complicates the analysis. This type of experiment is being repeated using other restriction enzymes and shorter pulse labeling periods to see if the end segments are truly underlabeled. Also, very short pieces of pulse labeled DNA will be isolated, labeled _in vitro_ to increase their radioactivity and hybridized to Southern transfers of restriction enzyme digests of intact DNA. These pieces should hybridize to segments which are synthesized first along the growing chain.

If a DNA region appears to contain an origin of replication, then it will be cloned and the relevant DNA will be sequenced. Also, any region which appears to contain a signal sequence, such as a palindrome of GC nucleotides, will be sequenced to examine the degree and extent of the palindrome.

Structural Characterization of Integrated Viral Genomes

In order to characterize the stable association between host cell DNA and viral sequences, molecular clones of several newly integrated retroviral genomes were produced in either plasmid or bacteriophage cloning vehicles using approved recombinant DNA techniques and were extensively studied using electron microscope heteroduplex methods. Retroviruses provide a unique and important system for such studies since exogenously acquired genomes are integrated at a specific site in the viral genome, but at a large number of sites in cellular DNA. The major objective of these studies has been the application of physical and biochemical techniques to assess the influence of flanking cellular sequences on subsequent viral function and to define in molecular terms these events which take place during integrative recombination.

Several distinct classes of viruses (including the lambdoid and mutator phages of _E. coli_, the papovaviruses and the retroviruses) form stable associations with their hosts by the integration of a copy of the viral genome into host cell DNA. However, the degree of specificity in each process appears to range from the case of lambda phage, in which a specific site in viral DNA recombines with a specific homologous site in bacterial DNA, to the case of the papovavirus, simian virus 40, in which the joining of host and viral DNA appears to occur randomly within both genomes. It has been shown in a number of retroviral systems that exogenously acquired genomes are integrated at a specific site in the viral genome, but at a large number of sites in cellular DNA. In order to investigate more closely this association between host cell and viral sequences, molecular clones of several newly integrated retroviral genomes were produced in either plasmid or bacteriophage cloning vehicles using approved recombinant DNA techniques and were studied using electron microscope heteroduplex methods. Electron microscope heteroduplex analysis is a sensitive method for detecting and mapping regions of sequence homology and nonhomology between related nucleic acid species.

The lack of an endonuclease Eco RI site in the AKR murine leukemia virus (MuLV) DNA genome was used to isolate integrated AKR MuLV DNA from productively infected mouse cells. Two clones (614 and 623) were shown to contain the entire 8.8kbp retroviral genome, flanked by nonviral cell sequences. No homology could be demonstrated between the nonviral cell sequences of the two clones, indicating that the integration sites were different. The DNA from both clones were highly infectious for mouse cells, although the specific infectivity and the titer of the derived, ecotropic MuLV was more than 10-fold higher with 623.

MCF 247 viral DNA, present in a plasmid recombinant, was compared by restriction endonuclease digestion and heteroduplex analysis with the AKR ecotropic viral DNA. Although MCF recombinants were originally constructed by the shotgun cloning of chronically infected mink cell DNA using the purified Eco RI "arms" of λgtWES.λB, the preparations contained exclusively viral DNA and no flanking cellular DNA sequences. The AKR recombinant (623), on the other hand, has previously been shown to contain the entire retrovirus genome as well as mouse DNA sequences which flank both termini. The MCF viral DNA measured 7kbp in length and appeared to be colinear with the 5' 70% of AKR viral genome. Of particular interest was the finding that when MCF DNA was annealed to the complete AKR insert (which also contained flanking cellular sequences) or a subclone that contained only the 3' AKR termini and adjacent mouse DNA sequences, circular heteroduplexes were observed which invariably contained a single, visible duplex projection. These 0.6kbp projections, which presumably result from hybridization of sequences at the 5'end of the MCF insert with those at or near the 3'end of the AKR insert, would further imply an unusual sequence inversion in these molecules. Although the fine structure and precise extent of homology in these projections are difficult to assess at present, these data are consistent with an unusual reiteration of viral sequences which might have arisen as a consequence of the integration of viral sequences into cellular DNA.

Similar structural analyses were performed on 3 molecular clones of newly integrated mouse mammary tumor virus (MMTV) genomes produced in bacteriophage lambda from a C3H mammary carcinoma derived cell line. While cloned fragments were shown to be colinear with the 3'end of MMTV viral sequences by restriction endonuclease, Rloop, heteroduplex and nucleotide sequence analyses, no homology could be detected beyond the virus-cell junction in any hostspecific sequences. However, within the cell-specific region of every integrated MMTV clone thus far examined, one or two prominent stem-loop structures were visible in the electron microscope. Certain length measurements of these structures reveal inverted repeats of 0.30-0.45 kbp with intervening sequences of 1.22.8 kbp. In at least one clone a stem-loop appears at or near the virus-cell junction. While the biological implications, if any, of these flanking sequence structures are not clear, they may represent a detectable feature of those regions of the host cell genome where frequent integration or recombination events occur.

We propose to exploit the potential of recombinant DNA technology for the detection and amplification of rare DNA sequences such as a single, integrated viral genome in order to 1) learn more about the specificity of various integration events, 2) assess the influence of flanking cellular sequences on the subsequent functioning of the viral genome and 3) to define in molecular terms those events that take place during integrative recombination.

Honors and Awards

Dr. Norman P. Salzman continued to serve on the Editorial Board of the Journal of Virology, the Scientific Board of the Coordinating Council for Career Research, and was reappointed to a three-year term on the Editorial Advisory Board, Biochemistry. He serves as Professorial Lecturer, Georgetown University School of Medicine, and was an invited participant at the Transforming Genes II Workshop, Villejuif, France.

Dr. Bernard Moss continued to serve as an Associate Editor of Virology and on the Editorial Boards of the Journal of Virology; of Antibiotics and Chemotherapy; and of Intervirology. In addition, he served as Chairman of the seminar on Transcription of the Genomes of DNA Containing Virus at the annual meeting of the American Society for Microbiology and was the Chairman of the Poxvirus Workshop held at Cold Spring Harbor Laboratories.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01AI 00123-14 LBV

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)
Viral and Eukaryotic RNA: Synthesis, Modification and Translation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

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SECTION
Macromolecular Biology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
9.25	6.25	3

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

Our primary objective is to determine the molecular events that lead to the selective transcription of DNA and subsequent processing of viral and eukaryotic messenger RNA (mRNA). Poxviruses provide a unique and important system for such studies since the enzymes needed for transcription and mRNA modification are packaged within the core of infectious virus particles. During the past year, several important advances have been made. For the first time, the RNA polymerase has been solubilized and purified from vaccinia virus particles. DNA recombinant technology has been applied to vaccinia virus and genome segments have been cloned in phage and plasmids. This has allowed us to determine the presence of 30 tandem repetitions of a 70 bp sequence, present near each end of the genome, which are presumably used for DNA replication. Several early mRNAs and polypeptides have been mapped within the long 10,000 bp inverted terminal repetition. Significantly, no evidence of RNA splicing has been found. Continued studies on the enzymatic mechanism of capping mRNA has led to the purification of a cap specific mRNA (nucleoside-2-)-methyltransferase from HeLa cells and capping enzyme from wheat germ. In addition, a transfer RNA (cytosine-5-)-methyltransferase was purified from HeLa cells and shown to specifically methylate cytidine residues within the transition sequence between the extra arm and the righthand loop of tRNA.

Major Findings

I. Post-transcriptional modification of eukaryotic RNA. Members of this research group previously identified the 5' terminal cap structure $m^7G(5')pppN$, consisting of a 7-methylguanosine (m^7G) linked by a 5' to 5' triphosphate bridge to one or two consecutive 2'-O-methylated ribonucleosides (N^m) present in viral and cellular mRNAs. The mechanism of cap formation was determined by purifying and characterizing the responsible enzymes from vaccinia virus. Subsequently, by isolating enzymes from HeLa cells, we demonstrated that cap formation of eukaryotic and viral mRNAs occur by similar, although slightly different, mechanisms. During the past year, we have extended this work by purifying a cap specific RNA (nucleoside 2'-)-methyltransferase from HeLa cells and an RNA guanylyltransferase (capping enzyme) from wheat germ. A transfer RNA (cytosine-5-)-methyltransferase was purified from HeLa cells and its substrate specificity was determined.

A. Purification and characterization of HeLa cell RNA (nucleoside-2'-) methyltransferase. A specific assay was developed for cap-specific RNA (nucleoside-2'-)-methyltransferase. The substrate for this assay was prepared by using purified vaccinia virus capping enzyme, synthetic poly(A, [α-^{32}P]GTP and S-adenosylmethionine (AdoMet). The substrate, $m^7G(5')\overset{*}{p}ppA(pA)$, and AdoMet were then incubated with enzyme fractions and the formation of $m^7G(5')\overset{*}{p}pA^m(pA)_n$ was detected by RNase T_2 digestion and high voltage DEAE paper electrophoresis. Using this assay, a specific RNA (nucleoside-2'-) methyltransferase was purified. Characterization of the enzyme is now in progress.

B. Purification of capping enzyme from wheat germ. Capping enzymes are extremely useful for specifically labeling the ends of RNA molecules. After receiving numerous requests for vaccinia virus and HeLa cell capping enzymes, which are difficult to purify in large quantities, we decided to attempt a purification from a more available source. Accordingly, wheat germ was chosen. Thus far, we have achieved a nearly 2,000 fold purification of the enzyme and have made a preliminary characterization of its catalytic activities which are more similar to the HeLa cell capping enzyme than to the vaccinia virus enzyme. That is, it has no associated RNA triphosphatase activity and requires a diphosphate terminated polyribonucleotide. Also, like the HeLa cell enzyme, it has no associated RNA(guanine-7-)methyltransferase.

C. Post-transcriptional modification of transfer RNA: purification and characterization of HeLa cell tRNA(cytosine-5-)-methyltransferase. Eukaryotic cells contain a large number of different tRNAs that have similar nucleotide modifications such as methylation. To better understand protein nucleic acid interactions, we have purified a tRNA (cytosine-5-)-methyltransferase from HeLa cells. The enzyme specifically methylates cytidine residues within the transition sequence between the extra arm and the righthand loop of tRNA. The ability of the enzyme to methylate different tRNAs in the same part of the molecule suggests that the enzyme recognizes a general or limited sequence. Since RNase T_1 oligonucleotides of tRNA were not substrates, appropriate secondary or tertiary structure is probably needed in addition to the proper general sequence. Such a feature would be advantageous in allowing a single enzyme to methylate a large number of different tRNAs.

II. Organization and transcription of the vaccinia virus genome. Vaccinia virus, a member of the poxvirus family, provides a unique system for studying the synthesis and processing of mRNA from a DNA genome; all enzymes necessary for the synthesis of functional mRNAare contained within the infectious virus particle. The goal of this project is to define the roles of all enzymes and protein factors involved in the regulation of transcription. During the past year, we have used DNA recombinant methods to clone portions of the vaccinia virus genome and to determine the organization of some early transcriptional units. Another major achievement has been the purification of the vaccinia RNA polymerase.

A. Tandem Repeats within the Inverted Terminal Repetition of Vaccinia Virus DNA. Last year, we demonstrated that the same 10,000 base pair (bp) sequence is present at both ends of the vaccinia virus genome. To facilitate further studies, this segment of the genome was cloned in coliphage lambda. This permitted more detailed analysis which led us to detect the presence of direct tandem repetitions of a 70 bp sequence. These tandem repetitions begin about 200 bp from the very end of the genome. There are 13 tandem repetitions, followed by a 435 bp unique sequence, followed by 17 tandem repetitions of the same 70 bp sequence. Thus, there are 30 repetitions of this sequence at each end of the genome. These sequences are not transcribed and we have proposed that they function to accelerate cyclization of single strands of DNA during replication.

B. Analysis and mapping of mRNA and polypeptides encoded within the inverted terminal repetition. Early vaccinia polypeptides encoded within the terminal repetition were mapped by cell-free translation of mRNA that was selected by hybridization to restriction fragments and separated strands of a recombinant lambda phage. The results, which were confirmed by hybrid arrest of translation, indicated that polypeptides of 7,500 (7.5K) 19,000 (19K) and 42,000 (42K) daltons map at approximately 3.2 to 4.3, 6.5 to 7.2 and 7.2 to 8.3 kbp from the end of the genome, respectively. mRNAs for the 42K and 7.5K polypeptides are transcribed towards the end of the genome whereas mRNA for the 19K polypeptide is transcribed in the opposite direction. The mRNAs coding for these polypeptides were resolved by agarose gel electrophoresis. No evidence for interrupted genes was obtained by electron microscopy or nuclease S1 analysis of RNA·DNA hybrids. Thus, RNA splicing, which has been demonstrated for DNA viruses that replicate within the nucleus of infected cells, does not appear to be involved in synthesis of these first vaccinia virus mRNAs to be examined.

C. Purification of the vaccinia virus RNA polymerase. The DNA-dependent RNA polymerase was extracted from vaccinia virions and purified to near homogeneity. The native enzyme has a molecular weight of approximately 500,000 and can be dissociated into polypeptides of 140,000, 137,000 37,000, 35,000, 31,000, 22,000 and 17,000 daltons. Activity is dependent on a single-stranded DNA template, all 4 ribonucleoside triphosphates, and Mn^{2+}. The vaccinia virus RNA polymerase can be differentiated from prokaryotic and eukaryotic RNA polymerases by its resistance to rifampicin and α-amanitin, respectively.

Publications

Gershowitz, A., and Moss, B. Abortive transcription products of vaccinia virus are guanylylated, methylated and polyadenylylated. J. Virol. 31: 894-853, 1979.

Cooper, J. A. and Moss, B. Translation of specific vaccinia virus RNAs purified as RNA·DNA hybrids on potassium iodide gradients. Nucleic Acids Res. 6: 3599-3612, 1979.

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Keith, J. M., Winters, E. M. and Moss, B. Purification and characterization of a HeLa cell transfer RNA(cytosine-5-)-methyltransferase. J. Biol. Chem. 255: 4636-4644, 1980

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00124-11

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Replication of the Parvovirus KRV

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator - Dr. Lois A. Salzman, Research Biochemist, LBV, NIAID

Other: Ms. Phyllis Fabisch, Microbiologist, LBV, NIAID

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SECTION
Molecular Structure Section

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have been studying the specific biochemical mechanisms involved in the replication of the autonomous parvovirus KRV in a rat nephroma cell line. We have studied transcription of the single-stranded KRV DNA genome both in vivo and in vitro in isolated nuclei. The major viral specific RNA synthesized in both studies is 19 to 22 S and represents a transcript of 60 to 75% of the viral genome. The 5' terminus of the RNA is probably initiated and capped in vitro by the sequence $m^7G(5')ppp(5')A$. We have mapped the RNA using Southern blotting and R-loop technique and find that the KRV mRNA maps from 0.38 to 0.98 on the viral DNA strand. Viral specific RNA transcripts are also found the full length of the viral genome. We hope to determine if post-transcriptional processing occurs and the mechanisms involved. We also plan to use the purified viral mRNA in a cell free protein synthesized system.

We have also completed our study of the 3'terminus of the viral DNA. The nucleotide sequence of the 3'terminus supported a hairpin configuration of the DNA which enables the single-stranded DNA to be self priming during synthesis of the double-stranded replicative intermediate. We are now sequencing the 5'terminus of the DNA to determine its configuration and to isolate a protein suspected to be associated with the 5'terminus if there is a protein associated with the 5'terminus.

Project Description

Parvoviruses are the smallest DNA containing viruses found in vertebrates. They infect a wide variety of species including man and moth. The parvoviruses are classified as autonomous or defective depending on whether or not they require a helper virus for replication. The virions replicate best in rapidly dividing cells and produce a wide variety of effects in insects, animals and cells in culture. The feline parvovirus has been linked to the naturally occuring disease feline panleukopenia. Within the past year the causative agent of an easily transmitted and rapidly fatal disease of dogs has been identified as a canine parvovirus. A vaccine offering protection against the canine parvovirus will be widely distributed within the year. Rodent parvoviruses can produce fatal infections and teratogenic effects in fetal and newborn hamsters. Other parvoviruses will probably be identified with disease states in the near future. Parvoviruses are able to persist for long periods of time in a latent state and to inhibit homologous and heterlogous virus infection. The viruses can also exhibit antimitotic activity and interfere with natural or virus-induced tumorigenesis.

We have continued our studies of the parvovirus, KRV, becuase of its genetic simplicity and the small size of the virus gene. KRV like the other parvoviruses possesses a single-stranded DNA genome (1.6×10^6 daltons or 4500 to 5000 bases) with sufficient information to code for only a few proteins. The transcription process may be very simple and involve only a few messenger RNAs. It is probable that the viral DNA replication and transcriptional components are carried out in large part by host functions. Thus, we potentially can gain insight into viral functions, host functions and the interaction between virus and host.

In the past year we have concentrated our efforts on further study of the transcription of the viral genome both in vivo and in vitro and to complete the nucleotide sequence of the 3' and 5'terminus of the viral DNA.

In our studies of the in vivo transcription of KRV, we have utilized nuclei isolated from rat nephroma cells infected with KRV. We have isolated viral specific transcripts from the infected cells as early as 8 h after infection. The predominant viral RNA species is 19-22S and represents a transcript of 60-75% of the viral single-stranded DNA. A viral RNA transcript represent ing 95-100% of the viral genome can also be detected. On the basis of α - amanitin inhibition studies, synthesis of the KRV transcript is believed to be mediated primarily by cellular RNA polymerase II. Over 65% of the viral-specific RNA in the isolated nuclei is polyadenylylated on the 3' terminus. The 5' terminus of the RNA is capped *in vitro* by the sequence $m^7G(5')ppp(5')A$. Incorporation of $\beta - {}^{32}P$ ATP into the 5' cap sequence suggests that initiation of viral RNA synthesis occurs in the infected isolated nuclei. We hope to study further *in vitro* synthesis of viral specific RNA by lysing the nuclei and utilizing this cell free system to determine the components required for viral transcription.

We found two years ago that the single-stranded DNA from KRV can serve *in vitro* as a self-primer for the synthesis of a complementary linear viral DNA strand. A double stranded viral DNA molecule has been proposed as an intermediate in the replication of the viral genome. Little is known about the replication of single-stranded linear DNA in a eukaryotic cell. Because of the importance of the 3' terminus of the viral DNA in self priming viral DNA synthesis and possibly in transcription we sequenced the 3' terminal nucleotides. This analysis has lead us to propose a 3' terminal hairpin structure with secondary structures and the nucleotide sequence of the DNA which at least *in vitro* serves as the origin of replication of the complementary strand. We are currently sequencing the 5' terminus of the viral DNA which appears to have a unique structure and possibly an associated protein.

In vitro studies of RNA transcription have complemented the in vivo studies and extended our knowledge of viral transcription. We have isolated from the polysomes of infected cells two major viral specific RNAs sedimenting in sucrose gradients at 21S and 12S. The 21S RNA has a molecular weight of approximately 1.05×10^6 and would represent a transcript of 65% of the genome. The smaller 12S RNA has a molecular weight of 0.3×10^6 and would represent a transcript of 20% of the genome. Both of these DNAs are transcribed from the viral strand of the viral DNA. No functional viral RNAs have been found which hybridize with the complementary strand of the viral DNA. Two techniques have now been used to map these mRNAs to the viral genome. We have mapped the fragments of several restriction enzymes to the KRV viral genome. Using the mapped restriction fragments, the major cytoplasmic viral RNA (21 S) has been mapped to the viral genome using the "Southern" blotting technique. This has been complemented by Electron Microscopy of "R loops" or RNA-DNA duplexes. Both techniques indicate that the 21S viral nRNA maps from approximately 0.38 to 0.98 on the viral DNA strand. Looking at total cellular RNA we can find viral specific transcripts the full length of the viral genome. We hope to study this transcript further to determine posttranscriptional processing occurs by simple cleavage or splicing and what regions are not transcribed into mRNA.

Publications

Salzman, L. A., Fabisch, P. and Wali, T. Parvovirus mRNA synthesis in isolated nuclei. J. Virol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00125-11 LBV

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Mechanisms of Viral DNA Replication, Transcription, and Integration

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	John A. Thompson	Staff Fellow	LBV, NIAID
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NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
10	8	2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The structure of SV40 mRNA molecules has been determined by preparing cDNA copies of the viral mRNAs using reverse transcriptase. Characterization of transcripts formed when SV40 DNA is transcribed by purified RNA polymerases has provided the location of promoters of RNA transcription. The role of viral proteins as regulators of transcription has been studied by in vitro transcription of nucleoprotein complexes.

Papovaviruses

Transcription and Replication of SV40

During viral infection, the virus enters the cell, and in the case of SV40, the DNA viral genome is transported to the nucleus. It is not clear what fraction of the protein which is present in virions remains associated with the viral DNA within the cell. Our current understanding of the infectious cycle indicates that transcription of the viral DNA is one of the earliest events, and that the viral DNA which is used as the template is associated with proteins. The precise nature of the nucleoprotein complex which is the functional transcription template is not defined. The enzyme which synthesizes this early viral RNA is the cellular RNA polymerase II. This is the enzyme which also synthesizes cell messenger RNA. We have continued our studies to define the mechanism of transcription and to understand how the synthesis of different populations of RNA are regulated during the infectious cycle.

Simian Virus 40 (SV40) provides an excellent model system for investigating the process of transcriptional regulation in eukaryotic cells, since some properties of viral transcriptional complexes and mRNA molecules have been determined. In addition, the entire nucleotide base sequence of SV40 DNA has been determined and like most eukaryotic chromatin, during the SV40 infection cycle, cellular histones H_2A, H_2, H_3, and H_4 are bound to the viral DNA.

Localization of RNA Polymerase Promoters on SV40 DNA

Transcription of SV40 DNA by the Escherichia coli RNA polymerase has been investigated as a model system for the development of methods applicable to the study of the mechanisms of transcription by the mammalian RNA polymerase II. E. coli RNA polymerase binding sites have been previously investigated by electron microscopy. Except for one binding site, their location on the SV40 DNA map is only approximate.

While E. coli RNA polymerase recognizes specific promoter sites, it does not recognize specific termination sites on SV40 DNA, and so RNA molecules that are formed are not of a defined size. We have developed a method that allows for the production of discrete RNA species. Preformed RNA polymerase-SV40 DNA transcriptional complexes are cleaved with a "single-cut" restriction endonuclease (Bam HI, Eco RI). These cleavages generate termination sites at precisely defined locations. The "run-off" RNA subsequently synthesized on these linearized complexes can be resolved into discrete bands by gel electrophoresis. Fifteen major initiation sites have been identified using this method. The determination of the size of each of these RNA species and of the direction of their transcription has enabled us to construct a detailed map of the promoters recognized by the E. coli RNA polymerase on SV40 DNA.

The determination of the sequence of the 5' ends of some of the transcripts initiated is presently being carried out and the precise location of these promoters will allow us to compare the DNA sequences upstream from these sites and provide a better understanding of the nature of the interactions between the RNA polymerase and the DNA.

Studies with eucaryotic RNA polymerase II

The studies with *E. coli* RNA polymerase have served as an initial guide in our studies with mammalian RNA polymerase II. Highly purified preparations of RNA polymerase II, isolated from calf thymus, are being utilized to transcribe linear, relaxed (nicked) and superhelical SV40 DNA in *in vitro* transcription systems. With each of these templates, α-^{32}P-UTP is incorporated into discrete products. However, the ^{32}P label products produced with linear and relaxed (nicked) DNA are not "classical transcripts"; instead these products appear to be covalent aducts of RNA and DNA. At present the possible significance of these aducts is being investigated. In addition, RNA products produced from superhelical DNA are being characterized with regard to initiation and termination sites. Once the interaction of RNA polymerase II and DNA have been worked out in this relatively simple system, it will be possible to test the effect of various cellular factors on the specificity of transcription.

Transcriptional Properties of SV40 Nucleoprotein Cores

During the past year, we have been characterizing what appears to be a "chromatin activation" process that occurs during SV40 virion maturation. Two distinct SV40 virus particles are produced during a lytic infection cycle. The initial viral particle (immature) is found only in the infected cell nuclei and is a precursor to the mature viral particle, which is released from the infected cell. Analysis of the two virion populations indicate that both have a sedimentation value of 240S, a density of 1.33 g/cc and are sensitive to *in vitro* EGTA-DTT dissociation, indicating similar capsid structure. However, when the viral chromatin packaged inside the virion is examined, significant differences appear. The DNA-protein complex of the immature virus particle has a sedimentation value of 75S and contains cellular histones H_1, H_2A, H_2B, H_3 and H_4. Micrococcal nuclease digestion of immature virion chromatin gives a typical nucleosomal DNA ladder pattern indicative of higher order chromatin structure. The rate of in corporation of ribonucleoside triphosphates into RNA with the immature chromatin particle is 5% of that obtained with SV40 form 1 DNA.

The 110S chromatin particle (SV40 core) packaged inside the mature SV40 virion contains acetylated histones H_2A, H_2B, H_3, H_4. No H_1 can be detected in the mature virion. In place of H_1, viral proteins VP_1 and VP_2 are found associated with the chromatin particle. Micrococcal nuclease digestion of mature virion chromatin results in only submonomer nucleosomal DNA bands, indicative of a loose association of nucleosomes with the viral DNA. Transcriptional studies performed with SV40 cores indicate that they are potentially active transcriptional complexes. The transcriptional activity of these complexes is extremely high and is similar to the activity obtained with purified SV40 F1 DNA; 20 fold higher than the rate observed with immature virion chromatin. Control experiments demonstrate that only when VP_1 and VP_2 are associated with the mature virion core is the transcriptional rate high; i.e. removal of VP_1/VP_2 to generate a DNA-histone (minichromosome) complex results in a decreased transcriptional efficiency. These results suggest that the viral proteins play an important role in the "activation" of the nucleohistone complex.

The SV40 chromatin modifications which appear to occur during viral maturation, i.e. histone acetylation, loss of histone H_1 increased sensitivity to DNAse 1, and association of non-histone proteins are all characteristic of the transition from transcriptionally inactive to active chromatin in eukaryotic cells. Further analysis of the chromatin transition and structure of the mature SV40 nucleoprotein core should provide an excellent system for analysis of eukaryotic RNA transcription.

Site Specific Mutagenesis

The purpose of the project is to define sites at the nucleotide level that control DNA replication and transcription of SV40. Purified DNA from SV40 has been nicked and gapped at a site known to contain signals for the two previously mentioned functions. This DNA served as a substrate for chemical mutagenesis that converts cytidine to uridine specifically in single-stranded nucleic acids. The chemically modified DNA has been inserted into a bacterial plasmid for the isolation and amplification of individual molecules. Specific chemical alterations in the DNA will be correlated to an altered function in the life cycle of SV40.

Publications

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00126-0 7LBV

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Restriction Enzyme Analysis of Vaccinia Virus DNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator: Frank DeFilippes, Research Physicist LBV NIAID

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1	1	0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of the project is to study the organization of genes in large DNA molecules obtained from animal viruses. The production and ordering of unique segments of vaccinia virus DNA provide a physical map of the genome. Physical maps have been determined for several different restriction enzymes including SalI, HindIII, XhoI, KpnI, SmaI and BglI. The last two enzymes are very useful since they cleave the DNA into five easily separated segments from which other restriction enzyme maps can be determined.

Experiments designed to locate the origin(s) of viral DNA replication by electrophoretic analysis of restriction nuclease digests of pulse labeled viral DNA have been initiated. At early times after infection, viral DNA is present in the nucleus and cytoplasm of infected HeLa cells. Early experiments indicate that HindIII segments A, B and C are underlabeled in full size molecules. This result is being examined with other restriction enzymes and conditions.

Viral DNA segments are being cloned in plasmids with the aim of doing sequence determination on the region containing the origin of replication and any possible signal elements in the viral genome.

PHS-6040
(Rev. 10-76)

Project Description

Restriction enzyme digests of vaccinia virus DNA usually produce 15 or more unique segments which are separated by agarose gel electrophoresis. The large number of pieces makes it difficult to order the segments on a physical map. The restriction enzyme BglI, however, cleaves vaccinia DNA into only four large pieces A, B, C and D, with masses of 72, 28, 14 and 7 X 10^6 daltons. These segments can be separated by sucrose gradient centrifugation and they can be recovered in 100 mg quantities with less than 20% cross contamination from a single sedimentation run. Each of these four segments is digested with a second restriction enzyme, such as XhoI, and the electrophoretic pattern of the segments formed by the double digest is compared with the pattern obtained by digestion of intact DNA with Xho alone. This procedure identifies those Xho segments which are contained intact in each Bgl segment. These latter segments are large so they are usually cleaved at several sites by a second restriction enzyme. Since the order of BglI segments has been determined as C, D, B, A, analysis of the double digest gives an approximate location of many of the Xho segments. BglI digests can be treated with SmaI which cleaves only the A segment into two pieces of 48 and 24 X 10^6 daltons. These two enzymes divide the vaccinia genome into five large pieces into which all other segments can be grouped. Using these two enzymes and additional techniques, physical maps of SalI, XhoI, HindIII and BglI have been completed and a map for KpnI has almost been finished.

BglI cleavage leaves a protruding 3' end. Duplex DNA has been labeled at this site with either $dNTP^{32}$ or $rNTP^{32}$ by using terminal transferase in the presence of Co^{++}. Restriction segments with several labeled nucleotides at one end have been used for mapping by a partial digestion method. BglI segments can be sequenced by labeling with CTP^{32} or GTP^{32} and then removing all but the terminal P^{32} nucleotide with ribonuclease.

Since sequence analysis is facilitated by having larger quantities of DNA available, some of the smaller segments of vaccinia virus DNA are being cloned in an _E. coli_ plasmid system. The plasmids are pBR327, a deletion mutant of pBR322, and pBR328, a deletion mutant of pBR325. SalI segment H has been cloned in pBR328 and we are currently trying to clone all or part of SalI segments C, D and E and XhoI segments C and D in pBR328, since these segments are hard to separate by agarose gel electrophoresis. Both Sal segments C and H have BglI sites.

HeLa cells have been labeled with 30 min pulses of ^3H-thymidine at different times after infection with high multiplicities of vaccinia virus to try to locate the origin of viral DNA replication. With these conditions, viral DNA synthesis peaks at 2.5 hours after infection. From 2 to 3.5 hours post infection, alkaline and neutral sucrose gradient sedimentation runs show labeled molecules of different size with less than 10% of the DNA in full length molecules. Electrophoresis of HindIII digests of these rapidly labeled full length molecules isolated from the cytoplasm shows a distribution of counts which is similar to the normal pattern. There are two additonal peaks, one located between segments F and G and the other between segments I and J. Also, the end segments B and C are underlabeled which is consistent with a region where replication begins. HindIII digests of full length DNA isolated from nuclei of cells infected 2.5 hours earlier also gives a distribution of label similar to the usual pattern except that peaks A ad B are underlabeled and the background level of counts is higher than that found for the cytoplasmic DNA. The labeled viral DNA isolated from the nucleus is clearly contaminated with cellular DNA and this complicates the analysis. This type of experiment is being repeated using other restriction enzymes and shorter pulse labeling periods to see if the end segments are truly underlabeled. Also, very short pieces of pulse labeled DNA will be isolated, labeled in vitro to increase their radioactivity and hybridized to Southern transfers of restriction enzyme digests of intact DNA. These pieces should hybridize to segments which are synthesized first along the growing chain.

If a DNA region appears to contain an origin of replication, then it will be cloned and the relevant DNA will be sequenced. Also, any region which appears to contain a signal sequence, such as a palindrome of GC nucleotides, will be sequenced to examine the degree and extent of the palindrome.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00127-13

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Structure and Function of Genetic and Protein Components of Defective Parvoviruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	James Rose	Section Head	LBV NIAID
Other:	John Janik	Research Associate	LBV NIAID
	Richard McPherson	Research Associate	LBV NIAID
	Er-Chung Wang	Visiting Fellow	LBV NIAID
	Marilyn Huston	Microbiologist	LBV NIAID
	Edwin Sebring	Research Chemist	LBV NIAID
	Frank Koczot	Research Biologist	LBV NIAID
	Claude Garon	Research Microbiologist	LBV NIAID
	James Garrison	Bio Lab Tech (Biochem)	LBV NIAID

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Biology of Viruses

SECTION
Molecular Structure Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 6.8	PROFESSIONAL: 5.7	OTHER: 1.1

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Main objectives in studying these defective human parvoviruses (AAV) are to (i) define specific biochemical mechanisms used in synthesizing their DNA, RNA and proteins, (ii) identify and characterize the helper virus-mediated step(s) required for their replication and (iii) relate biochemical findings to normal cellular processes as well as to potentials for selective interference with both AAV and Helper virus (adenoviruses, herpesviruses) infection. Among the methods used are nucleic acid hybridizations, analytical and preparative sucrose and CsCl sedimentations, molecular cleavage with restriction nucleases, gel electrophoresis, electron microscopy, DNA transfection, DNA cloning and microinjection of cells.

PHS-6040
(Rev. 10-76)

Project Description

(1) To gain insight into biochemical mechanisms involved in DNA replication, we have been studying AAV and adenovirus DNA synthesis in cell-free extracts. With respect to AAV, DNA replicative intermediates can be synthesized in soluble extracts prepared from infected cell nuclei. Conditions that yield *de novo* initiations of DNA synthesis as well as elongation of molecules already initiated *in vivo* have been defined. Analysis of the kinetics of DNA synthesis and of structural features of completed products continue to support our reported model for the self-primed replication of AAV DNA (Straus *et al*. (1976), *Proc*. *Natl*. *Acad*. *Sci*. *USA* 73, 742). Additionally, recent data suggests the mechanism by which adenovirus DNA synthesis is selectively suppressed during coinfections with AAV. We are now in the process of identifying and characterizing enzymatic and regulatory components that participate in viral and cellular DNA replication. An ultimate goal is to determine conditions needed for repeated reinitiations of DNA synthesis *in vitro*.

(2) AAV multiplication is dependent upon the expression of one or more early adenovirus genes (Straus *et al*. (1976), *J*. *Virol*. 17, 140; Richardson *et al*. (1980), *Proc*. *Natl*. *Acad*. *Sci*. *USA* 77, 931). In our current studies, DNA transfection experiments have been utilized to map the required adenovirus genes. Furthermore, functions provided by these genes have been determined by analysis of AAV macromolecular synthesis following transfection with selected adenovirus DNA fragments. We plan to investigate the specific biochemical interactions that are involved, since these early viral gene products carry out vital regulatory functions that serve as logical targets for selectively interfering with the process of viral infection.

(3) The adenovirus 72K DNA-binding protein is synthesized during the early period of adenovirus infection and appears to be a multifunctional element. It is required for viral DNA synthesis as well as for efficient expression of several late viral proteins. It also may be required for synthesis of all AAV structural polypeptides. Our present studies suggest that certain structural variations in the 72K protein can be related to the loss or gain of specific functions mediated by this protein. We plan to characterize more clearly the nature of such structural changes with the hope of gaining further insight into biochemical mechanisms utilized in the regulation of macromolecular synthesis.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Zo1 AI 00128-13 LBV

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Properties of Adenovirus DNA

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Claude F. Garon	Research Microbiologist	LBV	NIAID
	James A. Rose	Medical Officer	LBV	NIAID
Other:	James W. Garrison	Bio Lab Tech (Biochem)	LBV	NIAID

COOPERATING UNITS (if any)
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LAB/BRANCH
Laboratory of Biology of Viruses

SECTION
Macromolecular Biology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.2	PROFESSIONAL: .8	OTHER: .4

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major objective of these studies has been the application of physical and biochemical techniques to map and to define the structure of specific genetic regions in the genomes of both oncogenic and non-oncogenic human adenoviruses, with the ultimate goals of (1) relating these sequences to those biochemical activities which permit these viruses to influence or gain control of cellular functions (e.g., cell lysis and transformation) and (2) of defining basic mechanisms for regulation of genetic expression (e.g., initiation of DNA synthesis). A number of unique structural features of adenovirus DNA have been described which may have implications in viral replication or carcinogenesis, or both.

PHS-6040
(Rev. 10-76)

Project Description

Our initial work on the inverted terminal repetition found in the DNA of several human adenovirus serotypes also contained the observation that the length of these terminal sequences differed among the major serotype groups. A possible association between the increased length of the redundant segment and increased oncogenic capability was suggested. Therefore, we were interested in mapping the terminal nucleotide sequence of "highly oncogenic" adenovirus serotype 18 (Ad 18) and comparing this map with members of other major groups.

The structure of the DNA isolated from plaque-purified prototype Ad 18 has been analyzed by cleavage with restriction endonucleases, by electron microscope heteroduplex techniques and by nucleotide sequence analysis. A physical ordering of fragments was obtained by reciprocal digestion of isolated fragments and heteroduplex mapping. End fragments were identified by their susceptibility to exonuclease digestion and by the presence of covalently attached terminal proteins. Based on nucleotide sequence data, the inverted terminal repetition of Ad 18 is 165 nucleotides long and appears to be identical in sequence at both termini. The inverted terminal repeat in members of group B (Ad 3, 7) is reported to be 136 nucleotides in length while members of group C (Ad 2, 5) are 103 nucleotides long. Adenovirus serotype 12, also a member of the highly oncogenic group A, has a 162 nucleotide inverted terminal repeat that is not completely homologous to Ad 18. A plaque-purified variant of Ad 18 was isolated whose DNA measured approximately 4% longer in total length than the prototype strain. A structural comparison of the DNA of this isolate with that of the prototype strain revealed a restriction endonuclease cleavage pattern which appeared to differ only in the size of the terminal fragments. This finding was confirmed by electron microscope heteroduplex comparisons. Furthermore, the inverted terminal repetition of this isolate, which represents 3-4% of total genome length (or about 1300 nucleotides), did not replace any sequences that adjoin the inverted repeat of the prototype genome, nor did it contain any apparent internal molecular rearrangement. It is not known how these terminal repetitions originate, but it is apparent, however, that the mechanism involved can produce a variable but accurate inverted repeat. Since both the origin of replication and transforming activity have been mapped near the end of the genome, these isolates provide valuable reagents for the more detailed study of viral replication or carcinogenesis, or both.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00201-01 LBV

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Structural Characterization of Integrated Viral Genomes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator: Claude F. Garon, Res. Microbiologist, LBV, NIAID

Other: Nancy G. Schmidt, Biologist, LBV, NIAID
Malcolm A. Martin, Medical Officer, OSD, DRRU
Douglas R. Lowy, Sr. Investigator, D, NCI
Gordon L. Hager, Expert, LPVG, NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Biology of Viruses

SECTION
Macromolecular Biology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 2.7	PROFESSIONAL: 1.7	OTHER: 1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In order to characterize the stable association between host cell DNA and viral sequences, molecular clones of several newly integrated retroviral genomes were produced in either plasmid or bacteriophage cloning vehicles using approved recombinant DNA techniques and were extensively studied using electron microscope heteroduplex methods. Retroviruses provide a unique and important system for such studies since exogenously acquired genomes are integrated at a specific site in the viral genome, but at a large number of sites in cellular DNA. The major objective of these studies has been the application of physical and biochemical techniques to assess the influence of flanking cellular sequences on subsequent viral function and to define in molecular terms these events which take place during integrative recombination.

PHS-6040
(Rev. 10-76)

Several distinct classes of viruses (including the lambdoid and mutator phages of E. coli, the papovaviruses and the retroviruses) form stable associations with their hosts by the integration of a copy of the viral genome into host cell DNA. However, the degree of specificity in each process appears to range from the case of lambda phage, in which a specific site in viral DNA recombines with a specific homologous site in bacterial DNA, to the case of the papovavirus, simian virus 40, in which the joining of host and viral DNA appears to occur randomly within both genomes. It has been shown in a number of retroviral systems that exogenously acquired genomes are integrated at a specific site in the viral genome, but at a large number of sites in cellular DNA. In order to investigate more closely this association between host cell and viral sequences, molecular clones of several newly integrated retroviral genomes were produced in either plasmid or bacteriophage cloning vehicles using approved recombinant DNA techniques and were studied using electron microscope heteroduplex methods. Electron microscope heteroduplex analysis is a sensitive method for detecting and mapping regions of sequence homology and non-homology between related nucleic acid species.

The lack of an endonuclease Eco RI site in the AKR murine leukemia virus (MuLV) DNA genome was used to isolate integrated AKR MuLV DNA from productively infected mouse cells. Two clones (614 and 623) were shown to contain the entire 8.8kbp retroviral genome, flanked by nonviral cell sequences. No homology could be demonstrated between the nonviral cell sequences of the two clones, indicating that the integration sites were different. The DNA from both clones were highly infectious for mouse cells, although the specific infectivity and the titer of the derived, ecotropic MuLV was more than 10-fold higher with 623.

MCF 247 viral DNA, present in a plasmid recombinant, was compared by restriction endonuclease digestion and heteroduplex analysis with the AKR ecotropic viral DNA. Although MCF recombinants were originally constructed by the shotgun cloning of chronically infected mink cell DNA using the purified Eco RI "arms" of λgtWES.λB, the preparations contained exclusively viral DNA and no flanking cellular DNA sequences. The AKR recombinant (623), on the other hand, has previously been shown to contain the entire retrovirus genome as well as mouse DNA sequences which flank both termini. The MCF viral DNA measured 7kbp in length and appeared to be colinear with the 5' 70% of AKR viral genome. Of particular interest was the finding that when MCF DNA was annealed to the complete AKR insert (which also contained flanking cellular sequences) or a subclone that contained only the 3' AKR termini and adjacent mouse DNA sequences, circular heteroduplexes were observed which invariably contained a single, visible duplex projection. These 0.6kbp projections, which presumably result from hybridization of sequences at the 5'end of the MCF insert with those at or near the 3'end of the AKR insert, would further imply an unusual sequence inversion in these molecules. Although the fine structure and precise extent of homology in these projections are difficult to assess at present, these data are consistent with an unusual reiteration of viral sequences which might have arisen as a consequence of the integration of viral sequences into cellular DNA.

Similar structural analyses were performed on 3 molecular clones of newly integrated mouse mammary tumor virus (MMTV) genomes produced in bacteriophage lambda from a C3H mammary carcinoma derived cell line. While cloned fragments were shown to be colinear with the 3'end of MMTV viral sequences by restriction endonuclease, R-loop, heteroduplex and nucleotide sequence analyses, no homology could be detected beyond the virus-cell junction in any host-specific sequences. However, within the cell-specific region of every integrated MMTV clone thus far examined, one or two prominent stem-loop structures were visible in the electron microscope. Certain length measurements of these structures reveal inverted repeats of 0.30-0.45 kbp with intervening sequences of 1.2-2.8 kbp. In at least one clone a stem-loop appears at or near the virus-cell junction. While the biological implications, if any, of these flanking sequence structures are not clear, they may represent a detectable feature of those regions of the host cell genome where frequent integration or recombination events occur.

We propose to exploit the potential of recombinant DNA technology for the detection and amplification of rare DNA sequences such as a single, integrated viral genome in order to 1) learn more about the specificity of various integration events, 2) assess the influence of flanking cellular sequences on the subsequent functioning of the viral genome and 3) to define in molecular terms those events that take place during integrative recombination.

Publications:

Lowy, D.R., Rands, E., Chattopadhyay, S.K., Garon, C.F. and Hager, G.L. 1980. Molecular Cloning of Infectious Integrated Murine Leukemia Virus DNA From Infected Mouse Cells., Proc. Natl. Acad. Sci., USA 77: 614-618.

Hager, G.L., Chang, E.H., Chan, H.W., Garon, C.F., Israel, M.A., Martin, M.A., Scolnick, E.M., and Lowy, D.R. 1979. Molecular Cloning of the Harvey Sarcoma Circular DNA Intermediates: Initial Structural and Biologic Characterization. J. Virol. 31: 795-809.

Chan, H.W., Garon, C.F., Chang, E.H., Lowy, D.R., Hager, G.L., Scolnick, E.M., Repaske, R., Martin, M.A. 1980. Molecular Cloning of the Harvey Sarcoma Circular DNA Intermediates: Further Structural Analyses. J. Virol. 33: 845-855.

LABORATORY OF CLINICAL INVESTIGATION
1980 Annual Report
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Summary of Program
Laboratory of Clinical Investigation
October 1, 1979 - September 30, 1980

Michael M. Frank, M.D., Chief of Laboratory
and Clinical Director, NIAID

Introduction

The year of 1980 was an excellent one for the Laboratory of Clinical Investigation. The new organization of the Laboratory has been solidified and it has become clear that we have a coherent, solid clinical research program under the new general lines of organization. All of our initial staff recruitment is completed and our junior people are beginning to develop the experimental programs which will allow their work to proceed. Although we have fewer individual units in the Laboratory conducting clinical research at this time we have allowed our strongest programs to undergo considerable development. This development has led to a great expansion in research activity and, much more importantly, has allowed the research to become focused on the number of areas where the Laboratory of Clinical Investigation can make a major impact.

We now have three areas of general investigation. These are the general areas of clinical immunology, infectious disease and allergy. The staff of each of these areas is now relatively complete. Our program in humoral immunology is under the direction of Dr. Frank. This program includes Dr. Carl Hammer and Ms. Thelma Gaither as well as a number of clinical associates. Our humoral immunity program is complemented by a program in cellular immunology under Dr. Anthony Fauci. Dr. Barton Haynes has been a second senior member of this laboratory. The allergy program has continued to develop under Dr. Michael Kaliner as Chief of the Allergic disease center. During this year Dr. Dean Metcalfe was recruited to join the section. Dr. Metcalfe is one of our former clinical associates who left the Laboratory of Clinical Investigation some years ago to complete his training with Dr. K. Frank Austin. Dr. Metcalfe is an expert in allergy with a research interest in mucopolysaccharides and their mechanism of action. The general area he will develop is that of Food Hypersensitivity. Although Dr. Metcalfe's programs are only getting under way at the present time, we believe that the general area of Food Hypersensitivity is one in which a major impact can be made over the next 10 years that will affect the lives of many Americans. Thus, we are very pleased to have this program initiated at this time. Within the past several weeks Dr. Anna Maria Saavadra Delgado has joined the program on a parttime basis. This young internist/allergist from Puerto Rico will be working with us in developing the Food Hypersensitivity unit.

The Infectious Disease group now has representatives each of the major discipline areas of infectious disease. Thus, Dr. John Gallin is responsible for studies of the phagocytic cell and its contribution to host defense. He is particularly interested in patients with phagocytic cell defects as well as in patients with immunologic abnormalities and repeated infections such as those with cutaneous staphlococcal infections. Dr. John E. Bennett and Dr. June Kwon-Chung are responsible for the general area of mycology. Dr. Bennett is an internationally known clinician in this area and Dr. June Kwon-Chung is a Ph.D. expert in this area who works closely with Dr. Bennett in developing tests for fungal infections and in developing a better understanding of the physiology of these organisms. The area of virology is represented by Dr. Stephen Straus who has been developing his newly established program in viral disease and viral chemotherapy in a most satisfactory manner. The area of bacterial disease is further represented by Dr. Stephen Hosea whose major interest is in the of bacteremia and its consequences.

The three general areas of clinical immunology, allergy and infectious disease are thus each represented by individuals with National or international reputations and with strong basic and clinical programs in these areas. The details of these programs will be enumerated below. Nevertheless, it is fair to say that many members of the senior staff have achieved wide recognition for the quality of their research efforts and it is clear that the Laboratory of Clinical Investigation is making major strides in our understanding of clinical disease in man.

Research Accomplishments

CLINICAL IMMUNOLOGY SECTION

The Clinical Immunology Section has continued to make important advances in the areas of complement function, the mechanisms of immune complex disease, the pathogenesis of autoimmunity. In the general areas of complement function methods have been undergoing development for the preparation of large quantities of highly purified complement components for study in patients and in pathophysiologic situations (Hammer, Renfer, Gresham). Those studies are leading to the development for the first time of relatively simple methods for the preparation of large amounts of highly purified complement components. At the present time methods are under way for evaluating methods of storing those components once prepared because, in fact, even that technology has not been clearly established. The C3 opsonic site which allows materials to be phagocytized by phagocytic cells with C3 receptors has been under careful evaluation (Gaither, Hammer, Frank). It has become clear that this site is under very careful immunoregulatory control and various control proteins in serum regulate its expression. Assays are now ongoing for the levels of these control proteins and their mechanism of action. Moreover, the role of phagocytic cells in influencing the activity of these control proteins is under further careful evaluation (Gaither). It is hoped that as we begin to understand the creation and degredation of the opsonic site on foreign materials we will begin to understand why some agents are phagocytosed with greater difficulty and others more easily. Moreover, this will lead to the ability to evaluate whether phagocytosis is proceding correctly in clinical situations and to define new sets of diseases with one or more defect in the general phagocytic system.

Work in the Laboratory within the last year has also established the importance of C5 as an opsonin (Santaella, Gaither, Hammer, Frank). Although all the details of the way C5 interacts with phagocytic cells and other complement control proteins are not yet determined, it is clear that C5 augments the activity of C3 in producing phagocytosis when C3 is the limiting protein. Studies of Fc and C3 receptor function in vivo continued to procede within the Clinical Immunology Section (Berger, Gaither, Hammer). Working with a number of different collaborators listed in the actual research report it has been possible to start to determine the usefulness of this new test which was introduced into clinical medicine by the Clinical Immunology Section within the last several years. It has become obvious that this test will make a major impact on the way we think about autoimmune, rheumatologic, allergic and infectious diseases. Specifically, groups of patients are being defined with defects in receptor function for the first time (Frank, Hamburger, Lawley, Hall). These are the first patients ever discovered with reticuloendothelial system defects. Significantly the defects appear to be associated with situations in which immune complexes are deposited in tissues thereby causing clinical illness. Even more exciting is the recent discovery that defects in reticuloendothelial system Fc receptor splenic macrophages are present in groups of normal individuals known to have a high statistical chance of developing autoimmune disease. This raises important questions about the pathophysiology of these diseases (Lawley, Hall, Katz, Fauci, Frank). It may be that this observation will provide the breakthrough to understanding why some individuals develop autoimmune disease while most others do not. Groups of patients with systemic lupus erythematosus have been shown to have Fc receptor defects while more benign diseases such as mixed connective tissue disease, in general, are not associated with Fc receptor defects even though the patients may have circulating immune complexes.

As a further development of our studies of reticuloendothelial system function, Dr. Stephen Hosea and Dr. Eric Brown have been developing a model of bacteremia in which the role of reticuloendothelial system is examined with particular emphasis on the functions of antibody and complement in causing removal of bacteria from the circulation. These studies have paralled our studies of red cell clearance in autoimmune hemolytic anemia. The results have been very striking. It has been shown that with pneumococci the alternative pathway of complement activation, is of critical importance in causing bacterial clearance. It has been shown that the major effect of antibody is simply to allow complement activation to proceed and to augment the influence of complement in reducing bacterial survival in the circulation. It has also been emphasized that the major effect of antibody and complement is not to greatly increase the degree of RES sequestration of circulating bacteria, but rather to change the pattern of localization of the bacteria so that fewer bacteria are localized in the spleen and more bacteria are localized in the liver. The effectiveness of this alteration in bacterial sequestration patterns is directly related to the virulence of the bacterium. Organisms that are most easily opsonized for localization in the liver are the least virulent organisms for the animal. Organisms with the highest degree of localization within the red pulp of the spleen are the most virulent organisms for the animal. The role of splenectomy has been defined in this model. This is of great interest since patients with splenectomy are at greater risk than normals devleoping overwhelming pneumococcal sepsis. It has been shown in this model that the risk of developing sepsis is directly related to the type of pneumococcus. The most virulent pneumococci are the ones which most require splenec sequestration for effective blood stream sterilization. These are the same pneumococcal types that are most virulent for the animal and that show the greatest increase in virulence when the spleen is removed. Studies of the mechanism of bacterial opsonization are progressing quite satisfactorily and it is hoped that the molecular mechanisms of bacterial opsonization will be defined in precise terms within the next several years.

CLINICAL PHYSIOLOGY SECTION

The Clinical Physiology Section has been concerned with the second aspect of immunologic response in man--that of the cellular immune system. A large number of projects in both clinical and basic research areas have been ongoing during the year with many significant advances in the general area of immunoregulation as well as in clinical studies. In the area of immunoregulation the interaction between monocytes and B lymphocytes has been studied in detail (Stevenson, Fauci). A specific IgG antibody response was produced in vitro by the interaction of IgM containing immune complexes with cultured lymphocytes (Lane, Fauci). The role of the monocyte in regulating the activity and strength of a B lymphocyte response to specific antigens as well as to nonspecific mitogens has been explored and defined (Fauci). Current thinking suggests that macrophages and monocytes are important regulatory cells in the immune response. Specific monoclonal antisera have been developed with specificity for various monocyte macrophage antigens and methods have been developed for purifying populations of monocytes which limit the amount of activation that the monocyte undergoes during the purification procedure (Haynes, Stevenson, Fauci). Thus, cells which are much closer to normal circulating cells are now available for study in the laboratory. One aspect of the study of monocyte responses which is of particular interest was the demonstration that in monocyte induced suppressor activity there is an interaction between the monocyte and and Fc gamma receptor positive lymphocyte. This is the first demonstration of a monocyte T cell interaction in immunoregulation of normal human B cell reactivity (Fauci).

The development of hybridoma technology in the laboratory has made an enormous impact in clinical studies (Haynes, Fauci). The hybridoma studied in most detail thus far, reacts with 85% of perrpheral blood T lymphocytes in man. Most T gamma or so called suppressor cells do not contain on their surface the antigen which this antibody recognizes, however, a subset of Fc gamma receptor positive cells which are E rosette positive are detected by this antibody. This helps establish the fact that the T gamma lymphocyte is a heterogeneous population of cells and further delineates subpopulations of T gamma suppressor cells (Haynes, Fauci). It appears that this monoclonal antibody recognizes cells which act as helpers in mitogen induced B cell function. Other suppressor cells are positive for the antigen. Cells with this antigen on the surface do not mediate antibody dependent cellular cytotoxicity (Katz, Fauci). Nevertheless, not all helper cells have this antigen on their surface. The Sezary T cell is negative even though it maintains its functionally mature helper capability. Dr. Fauci and his colleagues have suggested that the Sezary cell lost this antigen during malignant transformation.

Adopting methodologies developed in other laboratories, Dr. Fauci's group successfully developed antigen specific T cell clones in man and has maintained these in cell culture. In addition to providing pure

T cell populations for functional assays, these cells provide a source T cell determinants without contamination from other cell types (Volkman, Fauci). Thus, they are particularly suitable for studies of T cell receptors.

Working on various aspects of cell purification, Dr. Fauci's group has developed methods using the recently described elutriator for preparing highly purified monocyte preparations and studying their functional capabilities (Stevenson, Fauci). A very important discovery from this group was the description for the first time of a primary immunodeficiency disease in man with a selective defect in NK activity in two patients with ChediakHigashi syndrome (Katz, Fauci). So called NK or natural killer activity may be very important in immune surveillance and these patients have a specific defect in this area. It is of great importance that patients with Chediak-Higashi syndrome are at high risk of developing tumors as a late complication of their disease. It is of particular interest that the defect in natural killer cell activity was partially restored by inducers of cyclic GMP in culture. Even more importantly while receiving ascorbic acid, a known stimulator of cyclic GMP, there was normalization of NK effector function in two patients with this disease. This became abnormal when ascorbic acid was discontinued (Katz, Fauci).

Dr. Fauci's group has also continued their exciting studies of pathophysiology and therapy of vasculitis including virtually all types of vasculitis in man. These studies have led to remarkably effective therapy for certain types of vasculitis including the systemic necrotizing vasculitis and other systemic vasculitities (Fauci, Cupps, Volkman). They have been less effective in the study in the treatment of cutaneous venulitis, however, this represents a much smaller clinical problem in man. The use of cyclophosphamide has led to dramatic reversions in disease toxicity and in striking regression of illness. The group is also characterizing and defining the hypereosinophilic syndrome a rare but often devastating disease. The clinical syndrome is being systematically defined and therapy is being systematically evaluated (Harley, Fauci).

ALLERGY AND HYPERSENSITIVITY SECTION

The Allergy Section has also had a very productive year. Major findings include identification of a number of the factors which influence mucous secretion in airways. These studies have led to studies of the factors which influence prostaglandin synthesis and secretion and a study of the effects of this prostaglandin formation on cellular function. In specific, it has been found that many prostaglandins increase mucous secretion (Marom). Interestingly, both aspirin and indomethacin (prostaglandin synthesis inhibitors) also increase mucous secretion. It appears that lypoxygenase products which are not inhibited by aspirin or indomethacin are in part responsible for this effect. About 50% of prostaglandin release by lung fragments following anaphylaxis in vitro is stimulated by histamine acting on these lung fragments and 50% of the release is independent of histamine. Several factors have been isolated from lung tissue and characterized which caused the synthesis of thromboxanes and prostaglandins from guinea pig lung tissue (Steel). Prostaglandins, therefore, appear to play a major regulatory role in allergic lung pathology and function. Factors that control mucous secretion by lung are of obvious great importance, since one of the prime difficulties that patients with asthma have is in difficulty with mucous secretion. Moreover, there are very few drugs which can regulate mucous secretion in a satisfactory manner.

Further work from the Allergy Section demonstrated that mast cell granules injected into the skin of rats and monkeys cause significant inflammatory reactions. Granules isolated with intact perigranular membranes cause a neutrophil-rich infiltrate apparent in two hours which peaked by 8 hours (Tannenbaum). An intense mononuclear infiltrate replaces the neutrophil infiltrate, subsequently peaking at 24 to 48 hours (Tannenbaum). Granules isolated free of membranes and free of mediators caused a less pronounced neutrophil response and a greater mononuclear infiltrate. The responsible factor was found to be associated with the granule matrix and this factor has been solubilized and partially characterized (Oertel). It has been termed inflammatory factor of anaphylaxis. The activity of this factor is clearly discussed by cortisone treatment of animals.

Studies have been ongoing as to the effect of calcium flux on mast cell function. Major increases of calcium into rat mast cells undergoing secretory events take place within seconds after stimulation. This calcium appears to play a major regulatory role in the mast cell as it does in many other cells. Ongoing studies are attempting to define this role in the secretory process (Donlon). A drug, Ameloride influences sodium calcium transport and causes dose dependent release of histamine from rat mast cells. Study of the action of this drug is providing greater insight into the role of calcium in the secretory process (Barr).

The effect of histamine on mast cells is under investigation. Preliminary evidence suggests the presence of H1 receptors but not H2 receptors on these cells (Westcott). The cells have also been examined for the presence of β adrenergic receptors (Donlon). 85,000 per cell, high affinity β adrenergic receptors have been identified. In addition, 320,000 per cell, low affinity receptors have been found. A significant increase in the number of receptors after degranulation was observed. Interestingly, the membranes of the intracellular granules also have β adrenergic receptors. It may be that these membranes fuse with the cell membrane leading to an increase in β adrenergic receptors on the cell.

Clinical studies have also proceeded in the laboratory. These studies are demonstrating the fact that subjects with asthma are significantly more sensitive than both allergic rhinitis and normal control subjects to α adrenergic stimuli (Shelhamer, Metcalfe, Smith). Patients with both asthma and allergic rhinitis have significant impairment of β adrenergic responses and have augmented parasympathetic function as compared with controls. It would appear that decreased β adrenergic response and increased cholinergic response is associated with the atopic state. Increased α adrenergic response is seen only in asthmatics. Interestingly, patients with cystic fibrosis, another chronic lung disease, have significantly greater sensitivity to both α adrenergic and cholinergic response than do normals and also have depressed β adrenergic response (Davis, Shelhamer). This is true in patients with these chronic lung diseases even in the absence of allergy. Thus, there may be an integral association between chronic lung disease, mucous secretion and airway response. Interestingly, hyperresponsiveness was found even in parents of patients with cystic fibrosis with no lung disease suggesting that these may be primary defects.

In another series of studies the presence of antibody against β adrenergic receptors in the plasma of asthmatics was detected and measured. Three out of four subjects who are the least responsive to therapy with isoproterenol had detectable antibody to β adrenergic receptors, perhaps explaining a portion of their decreased responsiveness (Ventor). Physiologic studies are also ongoing on the effect of histamine injection in patients (Summers, Sisker, Shelhamer). Surprisingly asthmatic patients required significantly more histamine for the induction of cutaneous flushing headaches, increases in pulse rate and pulse pressure. Thus, asthmatics are insensitive to histamine as compared to normal subjects. During the past year tests for urinary histamine were also developed and these will allow for further studies of histamine and its role in disease states (Myers).

CLINICAL MYCOLOGY SECTION

The Clinical Mycology Section has continued its studies of fungal disease in man. These studies have fallen into two general areas--studies of fungal diseases in man and studies of fungal physiology. One major effort has been involved with the isolation of aspergillis antigen in the serum of patients with aspergillosis as well as in experimentally infected animals in an attempt to allow for more rapid diagnosis of aspergillis. In other studies, studies have been performed on the effect of cryptococcal polysaccharide as a vaccine in normal volunteers. An antibody response to this polysaccharide has been demonstrated (Bennett and Henderson). Studies of the physiology of fungi includes studies of the chemical structure of the capsular polysacchride of serotype B of cryptococcus neoformans as well as studies of canavanine sensitivity of the four serotypes of cryptococcus neoformans and studies of the virulence of one of these forms in mice. Significant advances were made in the structural studies of the capsule with specific assignment of sugar linkages in the major capsular polysaccharide.

It was found that there was a difference in the sensitivity of various seratypes to canavanine and that this difference in sensitivity allowed for further division of cryptococcal types allowing for more easy identification of subtypes. A temperature sensitive and melanin lacking mutant of cryptococcus was isolated and found to be completely avirulent for mice and rats. Studies of this mutant and crosses with this mutuant to the wild type have shown that the mel locust plays a major role in the virulence of the isolate (Kwon-Chung, Bennett, Polacheck).

BACTERIAL DISEASES SECTION

Studies in the Bacterial Disease Section have continued to define the role of the phagocytic cell in host resistence to infection and the mechanisms behind specific diseases associated with frequent infection. There have been a number of major technical advances in the Laboratory which allowed for new methodology for quantitating leukocyte chemotaxis and bactericidal activity. Studies of chemotaxis can now proceed at ten times the previous rate of analysis. Also, methods have been developed for studying leukocyte spreading in a simple quantitative fashion. Studies in this laboratory have also led to the important observation that secretion of granules by neutrophils modulates chemotactic responsiveness. With limited exocytosis there is increased chemoattractant binding to the cell membrane which is due to the increased availability of chemoattractant receptors without altered affinity of binding. It may be that the membranes of granules actually become translocated to the cell membrane providing new cell membrane as well as new receptors at the cell surface. Following vigorous exocytosis of granules chemotaxis is inhibited. This inhibition is associated with decreased binding of chemotactic factors to the cell surface. Exocytosis of granules in vitro increases neutrophil adhesiveness and aggregation. This may be charge related and studies of neutrophil charge are proceeding in the laboratory. Deposition of calcium ion in the leading edge of cells in a gradient of chemoattractant demonstrated by this group using Electron Microscopy techniques raises the possibility that this may be of specific importance in cell motility and migration (Gallin and Seligmann).

During this year a patient with abnormal neutrophil adhesiveness and spreading associated with a missing plasma membrane glycoprotein was described. Studies were performed examining the effect of vitamin K derivitives on leukocyte function. It was found that vitamin K inhibits elicited membrane potentials of neutrophils, stimulates calcium release and inhibits the tyrosylation of the α chain of tubulin. Using fluorescent cyanine dyes and tritiated trimethyl phosphonium ions, chemoattractants and degranulating stimuli were shown to stimulate membrane potential changes in neutrophils. This technology allows one to study the efficiency of various chemotactic stimuli. The data suggest that degranulating stimuli modulate neutrophil locomotory responsiveness by alteration of neutrophil plasma membrane ion permeability or the concentration of intracellular ions. Use of these techniques in studies of neutrophils with patients with chronic granulomatous disease suggest that these neutrophils have a major abnormality in normal membrane function while other diseases are not associated with similar abnormalities. This abnormality is currently being characterized (Fletcher and Gallin).

Abnormal neutrophil function in cells with Chediak-Higashi syndrome was found to be associated with a 300 fold increase in a tyrosylation of the α chain of tubulin. This represents the first biochemical evidence for abnormal microtubule tubulin metabolism in a patient with phagocytic cell disfuntion (Nath, Gallin).

Studies of neutrophil function have suggested that two populations of neutrophils exist in man, those with easily demonstrable Fc receptors under a defined set of conditions and those without those receptors. These sets of neutrophils appear to be functionally dissimilar. Studies of these sets of neutrophils have suggested that both have normal complement receptors (Gallin, Frank, Cotton). This difference in reactivity of the two cell populations is nicely shown in studies of E. coli endotoxin administered to normal volunteers. During the period of neutropenia there is a marked increase in the percent of neutrophils without readily demonstrable IgG Fc receptors. Similarly, patients undergoing dialysis have an increase in the number of Fc receptor poor neutrophils. Those neutrophils with normal Fc receptor function appear to leave the circulation in these cases and probably perform most of the work of phagocytosis associated with neutrophil activity (Cotton, Gallin). Thus the neutrophils remaining in the circulation have decreased Fc receptors. A group of patients with high levels of IgE and repeated infections with Staphylococcus aureus and Candida albicans was studied. It was shown that these patients have specific IgG antibody to those organisms but not to other organisms suggesting the presence of an immunoregulatory defect. Moreover, it was found that histamine significantly affects leukocyte function. These findings provide further insight into the abnormal pathophysiology in patients with this group of diseases.(Berger, Gallin).

CLINICAL PARASITOLOGY SECTION

The assortment of human parasitic infections seen and studied by the Parasitology Section continues to increase with particular emphasis the past year on leishmaniasis, several variations of filarial infections, strongyloidias and schistosomiasis, including the new species *S. mekongi* from South East Asia.

Intensive studies were carried out on four patients with an unusual form of cutaneous leishmaniasis characterized by lesions which do not ulcerate, failure to respond to usual therapy and specifi anergy to the infecting organism. Blastogenic activity to leishmanial antigens was found to be due to a population of adherent suppressor cells (Petersen and Neva). Most of these patients were also found to have in vivo activity of B cells (Volkman, Petersen and Fauci). A surprisingly good clinical as well as parasitological response to carefully controlled local heat treatments was seen in one of the patients (Neva, Petersen and Corsey).

Blocking antibody, with characteristics of IgG were found to inhibit histamine release in patients with filarial and schistosome infections. This serum blocking activity was antigen specific since it was not effective in rag weed-induced histamine release and did not work with heterologous parasite antigens. This finding of blocking antibody to parasite antigen-induced histamine release may explain why patients chronically infected with parasites usually do not exhibit allergic manifestations (Ottesen).

IgE in the serum of cord blood from new borns is probably of fetal origin. This was found in babies born in India where their mothers had a 20% frequency of microfilaremia (Weil, Ottesen).

The first immunologic evaluation of patients with a new form of schistosomiasis, *S. mekongi*, in S.E. Asian refugees has been carried out. Arrangements have been made with a special protocol to treat 11 or 12 of the patients with a new drug, praziquantal (Ottesen and Nash).

The changes in immunological responsiveness in patients with *S. mansoni* after treatment with another new drug, oxammiquine are being followed in a well-defined Puerto Rican population with chronic infection (Hoffstetter and Ottesen).

Radioimmuno assays have been developed for measurement of antibody to two polysaccharide antigens from adult worm schistosomes. These tests are more sensitive and specific than the previous ELISA tests. For example, one of the tests can detect 1 to 10 nonograms per ml of antigen however, circulating serum antigen or antigen in urine could not be detected in heavily infected cases (Nash).

CLINICAL HYPERSENSITIVITY SECTION

This Section was discontinued this year with the departure of Dr. Kirkpatrick. Nevertheless it is important to note that a major breakthrough in the treatment of mucocutaneous candidiasis was made this year. In a blind study it was found that a new anti-fungal agent ketoconazole is highly effective in clearing lesions in patients with this disease. Many of these patients have a defect in cellular immune function and those patients always relapse in time because the underlying defect is still present. Nevertheless, this drug provides the first effective clinical control with a drug that is not highly toxic.

Administrative Changes

During the past year Dr. Charles Kirkpatrick left the Laboratory of Clinical Investigation to assume duties as Head of the Clinical Immunology at the Jewish Hospital in Denver. He also serves as Professor of Medicine at the University of Colorado School of Medicine. Dr. Barton Haynes recently left to become Associate Professor of Medicine at Duke University School of Medicine. In keeping with the general centralization of the facilities of the Laboratory of Clinical Investigation and with the decision that a small number of groups would grow somewhat, neither of these individuals will be replaced immediately.

A number of other administrative features of great import occurred last year. It was possible to develop a clinical immunology contract to allow patients admitted for clinical evaluation of chronic infections or chronic immunologic problems to have a sophisticated series of Laboratory tests done via a contract mechanism. Large numbers of patients are referred to the Laboratory of Clinical Investigation for screening. It was both impossible, from the point of view of time, and inappropriate from the point of view of activity, to have the research laboratory effort directed solely at evaluating these patients. Nevertheless, such evaluation was absolutely essential to determine which patients are suitable patients for research study. During the year 1980 a contract was established and set in motion to provide the sophisticated evaluative services required for the various laboratories in cellular immunology, humoral immunology and phagocytosis and phagocytic cell function so that patients could be screened effectively to determine whether they were suitable candidates for further research. Moreover, this year saw the initiation of what is hoped will be a fruitful collaboration between the laboratory of Dr. John Gerrin, and the Laboratory of Clinical Investigation. A young electron microscopist, Dr. Mark Friedman joined Dr. Gerrin's laboratory and will be collaborating with members of the Laboratory of Clinical Investigation on various electron microscopic aspects of the problems under investigation.

We hope that this will be the last year that we have to deal with the problems of space and equipment associated with waiting for the opening of the Ambulatory Care Research Facility. It is hoped that the next year we will see considerable progress in our ability to do clinical investigation in an outpatient setting and in settling our research laboratories into a laboratory configuration optimal to their functioning.

Honors and Awards

The members of the Laboratory of Clinical Investigation have received many honors and awards during the past year. Perhaps most noteworthy is the fact that officers of the two major clinical investigation societies in America are members of the staff of the Laboratory of Clinical Investigation. Thus, Dr. Anthony Fauci is currently President of the American Federation for Clinical Research and Dr. Michael M. Frank has recently been elected SecretaryTreasurer of the American Society for Clinical Investigation. Moreover, Dr. William Paul, Chief of the Laboratory of Immunology, is currently President of the American Society for Clinical Investigation. Thus of the many activities of the two major clinical research societies in America are being planned and reviewed within the Laboratory of Investigation and on the 11th floor of the Clinical Center.

Dr. Anthony Fauci received the Arthur S. Flemming Award as one of the 10 most outstanding Federal employees under age 40 presented by the Junior Chamber of Commerce. Thus, Dr. Fauci joins many prominant U.S. citizens in receiving this important recognition of accomplishment. Dr. Fauci is on the Postgraduate Education Committee of the American Academy of Allergy.

Dr. John Gallin was elected President of the "National Blood Club," a well-known group devoted to the study of hematology.

Members of the Laboratory continue to be very wellrepresented on the editorial boards of the major journals. Drs. Frank and Fauci are members of the editorial board of the Journal of Clinical Investigation. Drs. Fauci and Gallin are members of the editorial board of the Journal of Immunology. Indeed Dr. Fauci is the Section editor for Clinical Immunology in the Journal of Immunology. Dr. Fauci also represents clinical immunology in the American Journal of Medicine. Dr. Frank is on the editorial board of the Journal Blood as well as on the editorial board of the Journal Reviews in Infectious Diseases. Dr. Fauci also serves on the editorial board of three new journals. The Journal of Immunologic Pharmacology, Human Lymphocyte Differentiation and Clinics in Immunology and Allergy. Dr. John Gallin serves on the editorial board of Inflammation. Dr. John Bennett is on the editorial Board of the Journal of Infectious Diseases. Dr. Michael Kaliner is on the editorial board of the Journal of Allergy and Clinical Immunology.

Dr. June Kwon-Chung served as the Chairperson of the Medical Mycology Division of the American Society of Microbiology.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00043-15 LCI

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Immunology and Chemotherapy of Systemic Mycoses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.E. Bennett	Head, Clinical Mycology Section	LCI NIAID
OTHER:	D.K. Henderson	Guest Worker	" "

COOPERATING UNITS (if any)

S. Kim (FDA)
P. Pizzo (NCI)

LAB/BRANCH

Laboratory of Clinical Investigation

SECTION

Clinical Mycology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Galactomannan of Aspergillus fumigatus was measured in serum of patients with aspergillosis and experimentally infected rabbits.

Normal volunteers showed an increase in serum antibody to cryptococcal polysaccharide in response to vaccination with this antigen.

Project Description:

The general goals of the unit have been to study host defense in systemic mycoses and to improve diagnosis and treatment of these disorders.

Objectives:

1) Assess factors influencing galactomannanemia of patients and rabbits with aspergillosis.

2) Assess B cell tolerance in human cryptococcosis.

Methodology, Results and Significance:

1) Radioimmunoassay and ELISA methodology were applied to the detection of galactomannanemia in aspergillosis of experimental rabbits and of humans. The assays detected 20 ng/ml antigen in serum. Rabbits lethally infected with 5-300 x 10^6 spores were found to have 100-500 ng/ml of antigen 3-6 days after infection, using either RIA or ELISA. Surviving rabbits developed antibody to galactomannan by the tenth day post-infection. Two of six patients with invasive aspergillosis at autopsy had antigenemia with 90-500 ng/ml 1-4 days prior to death. Antigen was only detected when samples were diluted 1:4 and heated 90C 10 minutes, conditions which were found to release galactomannan from antigen-antibody complexes. Subsequent to the above work, the ultrasensitive radioenzymatic assay procedure has been adapted to measure galactomannan at concentrations of 1-5 ng/ml. These methods now are being used to study bloodstream clearance of antigen, antibody and antigen-antibody complexes in normal and infected rabbits. Future studies include determination of galactomannan binding to galactosyl receptors known to exist on macrophages and the influence antigalactomannan antibody has on attachment to this receptor. The hypothesis is that clearance of galactomannan depends, among other things, on availability of galactosyl binding sites in tissue, affinity of the galactomannan for these receptors, effect of antibody on ligand-receptor interaction, size of antigen-antibody complexes, and presence of complement components in the complex.

2) Normal human volunteers have been found to make an antibody response to 50 μg of C. neoformans polysaccharide, using either RIA or antibody class specific ELISA. The antibody response to C. neoformans and type III S. pneumoniae polysaccharide is being compared in volunteers and recovered, antibody-negative cryptococcosis patients to assess B cell tolerance. Future studies will include measurement of B cell receptors for C. neoformans polysaccharide in these two groups as well as assessing in vitro anticryptococcal antibody synthesis.

Publications:

1. Bennett, J.E.: Cryptococcosis, Mucormycosis, Aspergillosis, Candidiasis chapters. In Beeson, P.B., McDermott, W. and Wyngaarden, J.B. (eds.) Cecil Textbook of Medicine, 15th edition, W.B. Saunders, Philadelphia,

1979, pp 543-549.

2. Bennett, J.E.: Chapters - General Considerations (infections caused by higher bacteria and fungi), Actinomycosis, Nocardiosis, Cryptococcosis, Blastomycosis, Histoplasmosis, Coccidioidomycosis, Sporotrichosis, Moniliasis (Candidiasis), Aspergillosis, Phycomycosis, Other Deep Mycoses, and Superficial Mycoses Including Ringworm. In Isselbacher, K.J., Adams, R.D., Braunwald, E., Petersdorf, R.G., Wilson, J.D. (eds.) Harrisons Principles of Internal Medicine, 9th edition, McGraw Hill, New York, N.Y., ch. 150-162. In press.

3. Dismukes, W.E., Bennett, J.E., Drutz, D.J., Graybill, J.R., Remington, J.S., Stevens, D.A.: Criteria for the clinical evaluation of antifungal drugs. Rev. Infect. Dis. In press.

4. Bennett, J.E.: Treatment of cryptococcal, candidal and coccidioidal meningitis. In Remington, J.S. and Swartz, M. (eds.) Current Clinical Topics in Infectious Disease, Vol. 2, McGraw Hill, New York, N.Y. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 A1 00045-12 LCI

PERIOD COVERED

October 1,1979 to September 30,1980

TITLE OF PROJECT (80 characters or less)

Studies on the Interaction of Antibody and Complement on the Production of Immune Damage

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Frank	Head, Clinical Immunology Section and Chief	NIAID,LCI
Other:	C. Hammer	Senior Staff Fellow	NIAID,LCI
	T. Gaither	Resident Biologist	NIAID,LCI
	K. Katusha	Microbiologist	NIAID,LCI
	L. Renfer	Chemist	NIAID,LCI
	M. Santaella	Guest Researcher on IPA	NIAID,LCI
	H. Gresham	Medical Technologist	NIAID,LCI
	E. Brown	Clinical Associate	NIAID,LCI
	S. Hosea	Senior Investigator	NIAID,LCI
	G. Wirtz	Guest worker	NIAID,LCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Clinical Investigation

SECTION

Clinical Immunology Section

INSTITUTE AND LOCATION

NIH, NIAID, Bethesda, MD 20205

TOTAL MANYEARS: 5 1/12	PROFESSIONAL: 2 1/12	OTHER: 3.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒X(c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A sensitive functional assay has been developed which allows for the quantitation of the three control proteins in serum which regulate the activity of the opsonic C3b site on a cell membrane surface. Using this assay, it has been possible to determine the nature of the biochemical interactions which lead to C3b destruction. The ability of the various C3 fragments to bind to C3b receptors on neutrophils has been studied as has the ability of C5 to bind to the and C3d sites.

Methods have been developed for the large scale purification and eventual isolation of each of the 18 defined proteins comprising the human complement system. Complement components C3, C5, C7, and C8 have been subsequently purified in high yield to functional and biochemical purity. Complement components C1q, C1 esterase inhibitor, C2, C3INA, β1H, C4, C6 and C9 as well as the C5b,6 complex are not yet homogenous and protocols are in progress for their final isolation.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To develop a sensitive functional assay for the control proteins of the alternative pathway of human sera. To use this assay to study the mechanisms of destruction of the C3b opsonic site on a membrane surface. To then extend the methodology to evaluate the C3b opsonic site on bacterial, viral and fungal surfaces and to determine how function of these control proteins influences the pathophysiology of the infectious process. To examine the interaction of C5 with cell bound C3 and determine its contribution to opsonization.

As an adjunct to an intensive and detailed study of the role of antibody and complement in humoral and cellular reactions, we have continued to develop new methodology for the eventual purification and isolation of homogeneous preparations of each of the 18 defined proteins comprising the human complement system.

Methods Employed: Highly purified functionally active human C3 is prepared, radiolabelled, and used to form the complement intermediate site SAC43b on sheep erythrocytes. This intermediate is then incubated with highly purified control proteins or with dilutions of serum containing the control proteins. On further incubation with trypsin, there is release of the radiolabelled C3 fragment C3c from the erythrocyte surface, if the control proteins β1H and C3b inactivator have been functionally active. The biological activity, molecular weight and chain structure of the various cell bound C3 fragments, as well as of the released C3c fragment, can be followed at each step in the process of C3b degradation. The interaction of purified, radiolabelled C5 with these various sites and with neutrophil complement receptors is also examined.

A wide variety of standard and some novel physical biochemical and immunological techniques for the purification of proteins have been used to develop an efficient methodology for the purification of complement components.

Plasma pools and partially purified mixtures were fractionated and concentrated by the use of selective protein precipitating agents such as polyethylene glycol (PEG) and ammonium sulfate. Concentration and adjustment by solute exchange was also accomplished by the use of colloidion bags, Amicon concentration cells and by the use of a Millipore Pellicon Cassette System early in the purification protocol when adjustment of large (2-10 liter) volumes of material was required prior to application to chromatographic columns. Resolution of the mixture of complement components and other plasma proteins was accomplished by several sequential chromatographic steps including: ion-exchange resins (CM and DEAE Celluloses), molecular sieve filtration on agarose and dextran gels and by adsorption and differential elution from hydroxyl apatite, a form of insolubilized calcium phosphate.

PAGE in slab gels was also used as one method of ascertaining the purity of the final protein preparations, as well as for generating final stage material for monospecific antiserum production. Purity was also assessed by conventional immunoelectrophoresis and double diffusion techniques using antisera to known serum proteins. Sodium dodecyl sulfate-PAGE analysis was used for molecular characterization of the final product as to size and subunit structure and as an additional check on homogeneity of the final product.

Major Findings:

Incubation of EAC43b with β1H led to some decrease in immune adherence activity but no loss in C3b functional hemolytic activity and no change in the integrity of the α- or β-chains. Addition of C3b inactivator to the membrane-bound β1H-C3b complex led to a single cleavage of the C3b α-chain without release of the C3c fragment from the cellular site. This led to complete loss of C3b functional hemolytic activity. The α-chain cleavage led to loss but not complete disappearance of immune adherence reactivity. The action of both β1H and the C3b inactivator on cell-bound C3b led to the appearance of a trypsin-sensitive bond within the α-chain. Addition of trypsin led to a second cleavage of the α-chain and the release of the radiolabelled C3c fragment. In kinetic studies, the appearance of the trypsin-sensitive bond was shown to parallel the loss of C3b functional hemolytic activity. Kinetic studies demonstrated that both plasma and serum contain all of the ingredients for a C3b-cleaving system. By utilizing this sensitive radio-release assay, the functional titers of C3b inactivator in the serum of 15 normal individuals were found to be 11,310 ± 666 S.E. The mean β1H titer for 12 normal individuals was 20,600 ± 873. This assay can be used to quantitate the level of the trypsin-like enzyme as well. The assay provides a simple method to quantitate and evaluate the function of the C3b control proteins. C5 binding to C3b + C3d without subsequent hemolytic activation has been documented. This red cell bound C3-C5 complex promotes binding to neutrophils.

Using methods initially developed for the purification of C3;C5 and C6;C7 from separate plasma pools, respectively, we have consolidated our purification protocol to allow for the isolation of most all known complement proteins in a biologically active, homogeneous form from one large pool (2-10 liters) of human plasma. The basis for success in a protein purification scheme of this magnitude rests on the ability to obtain a large number of selective, highly resolved chromatographic pools early in the protocol in which biological activity has been stabilized. We have found that the use of specific protease inhibitors and agents which block complement activation is requisite for recovery of biological activity of many of the complement proteins. We have also demonstrated that in addition to the use of inhibitors in preservation of complement activity, the ability to proceed rapidly through the first ion-exchange step yields improved recoveries of biological activity for specific components.

In our current protocol, a series of initial steps (PEG precipitation, affinity adsorption of the PEG supernatant, low ionic strength adjustment and concentration followed by DEAE ion-exchange chromatography) results in resolution of the mixture of complement components such that eight distinct pools can be produced each of which contains no more than three complement components. Effectively, all of each component applied to the column is found within a pool at a functional level of 50 to 95% of starting values. Four complement components contained within four pools have been further purified in several stages to homogeneity. These are C3, C5, C7 and C8. Complement components C4, C6 and C9 are in a good state of purity and require only minor purification to obtain homogeneous preparations. C1q, C4B.P., C2, FB, C31NA, β1H and C1EI are being characterized for further purification.

Significance to Biomedical Research:

At present it is clear that complement activation plays an essential role in protecting the individual from infection with microorganisms. It is also clear that unregulated complement activation is a crucial component of autoimmune disease and regularly leads to tissue injury. Very little is known about how the complement proteins function in disease states however. The studies underway in our laboratory at present will make it possible to explore each of these questions in a systemic manner. They represent the overcoming of formidable methodologic barriers and are essential to further progress in these areas.

Proposed Course:

We plan to extend these studies to obtain a further understanding of the role of the proteins in the regulation of complement function in normal physiologic situations and in disease states. We plan to examine the role of control proteins in infectious states and in control against bacterial infection. In addition, we plan to determine the role of complement activation fragments in the development of clinical signs and symptoms of bacterial disease. We plan to further purify complement proteins and components so that we can study their metabolism and interaction in a variety of disease states.

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3. Gaither, T.A., Hammer, C. and Frank, M.M.: Studies of the Molecular Mechanisms of C3b Inactivation and a Simplified Assay for β1H and C3b Inactivator. J. Immunol. 123(3) 1195-1204.

4. Lawley, T.J., Moutsopoulos, H.M., Katz, S.I., Theofilopoulos, A.N., Chused, T.M. and Frank, M.M.: Demonstration of Circulating Immune Complexes in Sjögrens Syndrome. J. Immunol. 123: 1382-1387, 1979.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00046-12 LCI

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Pathogenesis of Delayed Hypersensitivity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. Michael M. Frank	Clinical Director, Lab Chief,	NIAID	LCI
Co-PI:	Dr. Eskild A. Petersen	Guest Worker,	NIAID	LCI
Other:	Dr. Aldina Barral	Fogarty Fellow	NIAID,	LPD
	Dr. Franklin D. Neva	Chief,	NIAID,	LPD

COOPERATING UNITS (if any)
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Denver, Colorado 80206

LAB/BRANCH
Laboratory of Clinical Investigation

SECTION
Clinical Mycology Section

INSTITUTE AND LOCATION
NIH, NIAID, Bethesda, MD 20205

TOTAL MANYEARS: 2	PROFESSIONAL: 2	OTHER:

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A) Patients with leishmaniasis were studied. Thus far the following data have been accumulated. Patients with diffuse cutaneous leishmaniasis show anergy to leishmania antigens. This anergy is antigen specific and is mediated by an adherent suppressor cell population. The defect can also be corrected by addition of prostaglandin synthetase inhibitors.

B) A murine model for studying transfer factor (TF) was developed. TF activity is dose dependent and proportional to spleen cell equivalent. TF is antigen specific and activity can be ablated by simultaneous administration with relevant but not irrelevant antigen. TF phenomena is time dependent.

C) A controlled clinical trial in mucocutaneous candidiasis has been conducted. The study showed that ketoconazole tablets taken orally for a 6 months period is highly efficacious in treating symptoms and signs of the disease but not curative.

Objectives:

1) Characterization of cellular immune responses in immunologically deficient patients with chronic infectious diseases.

2) Establish murine models for studying transfer factor.

3) Evaluate biology of transfer factor.

4) Control of candidiasis in patients with CMC.

Methods Employed

A) Leishmaniasis

Patients with diffuse cutaneous leishmaniasis from the Dominican Republic, patients with cutaneous leishmaniasis acquired in Panama and normal controls were studied.

Total number of T cells, subset of T cells, lymphocyte proliferation responses to mitogens and antigens as well as the effect of macrophage depletion and macrophage reconstitution *in vitro* were studied. A murine model for studying infection with L. brasilieisis has likewise been developed.

B) Transer Factor

We have developed murine models in inbred C57Bl/6 and Balb/c mice. Antigens employed were *Candida albicans* consisting of a sonicated serotype A, yeast phase suspension of heat-killed organisms. Soluble antigens were ferritin and PPD. Sensitization was done by either injecting candida antigen intradermally weekly for 3 weeks or by one subcutaneous injection in the tail with ferritin in CFA.

Spleen cells were obtained by gently forcing the spleen through 60 mesh stainless steel screens.

Transfer factor was prepared by repeated freeze-thawing spleen cells and dialyzing the lysates against distilled water. The dialysate was lyophilized and reconstituted with distilled water so that the material from 2 x 10^8 spleen cells was contained in each ml.

DTH reactions were studied by injection of 50-100 ug of antigen into the footpad and measuring the change in paw size at 24 and 48 hours. Feet were excised and histological scores were done in a blinded manner. The studies were designed in such a way that analysis on repeated measurement on the animals in each group could be performed and the variability with in and among animals be compared.

C) CMC

A double-blind controlled clinical trial of ketoconazole in chronic mucocutaneous candiasis was carried out. Important features of methodology for this study include strict criteria for defining failure at specific time intervals.

Major Findings:

A) Four patients, two males and two females, with DCL from the Dominican Republic were admitted to the Clinical Center, NIH and studied in detial. The patients ages ranged from 8 to 56 years. Each patient had had leishmaniasis for at least 5 years and three patients had previously received three courses each of treatment with pentavalent antimony without clearing of their disease. Lesions were extensive in patients 1-3 but minimal in patient 4. Three patients had elevation of IgG or IgM and all 4 patients had marked elevation of IgE. Delayed type hypersensitivity skin test responses to leishmanial antigens were absent in all 4 patients but 3 had skin test responses to other antigens. Total number of lymhocytes and of T and B cells were normal however subsets of T cells revealed T gamma to be low. No patients had circulating immune complexes as determined by Raji cell and Clq binding assays. Lymphocyte proliferative responses (LPR) to PHA and Con A were normal in all 4 patients. However 3 patients had absent LPR to leishmanial antigens but intact responses to other antigens such as candida, tetanus, PPD and SK/SD. Addition of indomethacin (1 μg/ml) but not alcohol control enhanced LPR to leishmanial antigens markedly with little effect on responses to other antigens. Passage of patients mononuclear cells over nylon wool columns with subsequent diminution but not depletion of macrophages likewise reconstituted the patients LPR to leishmanial antigens. No serum suppressive factor was found as LPR to leishmanial antigens were no different whether cultured in AB serum on autologous plasma. Control experiments were performed on mononuclear cells obtained from patients with acute cutaneous leishmaniasis, patients in reconvalesence, and normal non infected persons. Patients with uncomplicated leishmaniasis had marked LPR to leishmanial antigens where as normal control showed no responses and in no instances were antigen specific enhancement seen in either groups after indocin or column treatment. Thus, these studies demonstrated the presence of adherent suppressor cells in DCL which were speciifc for LPR to leishmanial antigens. The suppression was reversible _in vitro_ by indocin suggesting prostaglandin as a mediator of suppression. These studies offer a potential explanation for the selective immunosuppressive state in DCL as well as a possible modality for adjunct therapy.

B) Transfer Factor

1) Actively sensitized mice exhibit footpad swelling responses when tested with relevant antigens. The magnitude of responses is dependent on concentration of test antigen.

2) Passive sensitization with immune spleen cells and dialysates of immune spleen cells transfers sensitivity to relevant antigens. No footpad responses occurred in recipients of saline, non-immune spleen cells or recipients of serum, spleen cells or transfer factor from animals sensitized with other antigens.

3) There is a linear relationship between the log of the dose of transfer factor (in spleen cells equivalents) and magnitude of footpad swelling.

4) TF transfers FP swelling activity if antigen presented following a 24 hour interval.

5) No activity is transferred when TF and antigen are administered simultaneously.

6) Prior mixing of TF and antigen _in vitro_ will likewise abolish TF activity to relevant, but not irrelevant antigens.

7) Transfer factor and antigen presented together at time 0 will ablate TF activity. There is no footpad response to a test with the same antigen 24 hours later. However, if immune serum is also administered at 0, TF activity is still present.

C) CMC

Twelve patients with CMC of 3 or more years duration were assigned by random allocation to treatment with KTZ or placebo in a double-blind trial. Each of the 6 recipients of KTZ had remission of symptoms and marked regression of nucosal, skin, and nail lesions whereas only 2 of those receiving placebo had mucosal clearing and none had improvement of skin or nail disease. The clinical outcome in the KTZ group treated was significantly more favorable ($p \doteq 0.001$) than in the placebo treated group. The 6 patients who received placebo in the controlled trial were then treated with KTZ in an open trial and all had favorable responses. The average number of days of KTZ treatment which elapsed before improvement seen was: 6.0 ± 0.5 for mucosal lesions, 16 ± 2 for skin lesions, and 76 ± 13 for nail disease. One patient developed drug induced hepatitis after 6 months of treatment but this proved to be mild and reversible.

KTZ is highly effective treatment for chronic mucocutaneous candidiasis. In this small series of patients use of the drug was attended by only one instance of toxicity.

Significance

A) These experiments have demonstrated a potentially important mechanism by which patients with chronic infections becomes anergic and also offers a mode of possible therapeutic intervention.

B) Biology of transfer factor can now be studied in detail in an animal model and questions regarding biochemistry and possible therapeutic efficacy addressed.

C) Chronic mucocutaneous candidiasis can be treated successfully with an oral compound.

Proposed Course

A) Continue study patient with leishmaniasis in the Dominican Republic and at NIH.

B) Continue study

C) Long term effect of ketoconazole to be evaluated

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3. Petersen, E.A., Alling, D.W. and Kirkpatrick, C.H.: Treatment of chronic mucocutaneous candidiasis with ketoconazole, a controlled clinical trial. Annals of Internal Medicine, accepted.

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5. Petersen, E.A., Neva, F.A., Bogart, H. and Fauci, A.S.: Antigen specific suppresses monocytes in diffuse cutaneous leishmaniasis. Clin. Res. 357A, 1980.

6. Petersen, E.A., Greenberg, L. and Kirkpatrick, C.H.: Murine transfer Factor I, Transfer of cell mediated reactivity to particulate and soluble antigens. In preparation.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00047-11 LCI

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Clinical Studies on Patients with Known or Suspected Parasitic Diseases.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.'s:	F.A. Neva	Head, Clinical Parasitology Section, LCI, NIAID (dual position)
	T.E. Nash	Senior Investigator, Clinical Parasitology Section, LCI, NIAID (dual position)
	E.A. Ottesen	Senior Investigator, Clinical Parasitology Section, LCI, NIAID (dual position)
Others:	E. Petersen	Collaborative Researcher (IPA), LCI, NIAID
	T. Lawley	Medical Investigator, NCI
	G. Weil	Research Associate, LPD, NIAID
	R. Corsey	Electrical Engineer, Applied Clinical Section, BEIB, NIH
	D. Volkman	Clinical Associate, LCI, NIAID
	A. Fauci	Senior Investigator, LCI, NIAID

COOPERATING UNITS (if any) Institute of Dermatology, Santa Domingo, Dominican Republic (Dr. Bogaert); Tuberculosis Chemotherapy Center, Madras, India (Dr. S.P. Tripathy); Medical College of Madras, India (Prof. K.V. Thiruvengadem); San Juan Laboratory of CDC (E. Ruiz Tiben)

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☒ (a) HUMAN SUBJECTS ☒(b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) Immunologic studies were carried out on 4 patients with an unusual form of cutaneous leishmaniasis characterized by disseminated lesions, failure to respond to therapy and specific anergy to the infecting organism. Blastogenesis to leishmanial antigens could be restored by removal of adherent suppressor cells. Most of these patients also had in vivo activation of B cells. One of the patients had an excellent response to controlled local heat treatment. The serum of patients with filarial and schistosome infections was found to contain blocking activity to antigen-induced histamine release. The blocking activity had characteristics of IgG and was antigen specific. The demonstration of IgE in cord blood of Indian newborns was attributed to transplacental passage of IgE stimulated by filarial infection. Laotian refugee patients with a new form of schistosomiasis due to S. mekongi have been evaluated immunologically for the first time. Arrangements have been made to treat them with a new drug, pranziquantel. Immunologic studies were done before and after treatment with another new drug, oxamniquine. An improved radio-immuno assay was developed for detection of two polysaccharide antigens of schistosomes and antibody to them.

PHS-6040
(Rev. 10-76)

Professional Personnel, cont'd.:

Others:	M. Hofstetter	Research Associate, LPD,NIAID
	M. Barral	Guest Worker, LPD,NIAID
	R. Hussain	Collaborative Researcher (IPA), LPD,NIAID

Project Description:

This report covers the clinical investigations carried out by the three investigators in the Clinical Parasitology Section (Drs. Neva, Nash and Ottesen). Their experimental or non-clinical research is described in the Annual Report from the Laboratory of Parasitic Diseases. Therefore, there will be some overlap in reporting and in publications listed. The Clinical Parasitology Section provides consultative service in clinical parasitology to the Clinical Center and some members of the Section also participate as infectious disease consultants at the Clinical Center and at the Navel Medical Center. Some of the studies covered in this report were carried out in collaboration with investigators in the Dominican Republic, India and in Puerto Rico.

Pathogenesis and Immunology of Leishmaniasis. Through the collaboration of a group in the Dominican Republic we had the opportunity to study four patients at the Clinical Center with an unusual form of cutaneous leishmaniasis of the disseminated form (DCL). A small focus of this disease, with most cases being of the DCL variety, has recently been found in that country (Dr. Bogaert). DCL is characterized clinically by multiple lesions that do not ulcerate and are refractory to treatment, and specific anergy to antigens of the infecting organism, although complete immunological assessment of DCL patients is lacking. Specific anergy to leishmania antigens, by delayed skin reactions as well as by lymphocyte blastogenesis, was confirmed in 3 of the 4 Dominican patients. Blastogenic reactions could be restored in these cases by removal of adherent cells (Petersen and Neva). The same patients with evidence of immunosuppression were found to have *in vivo* activated B cells by hemolytic plaque assay (Volkman, Petersen and Fauci). Treatment with local heat using circulating water at 45°C, which resulted in skin temperatures of 41°C, was found to be remarkably effective both clinically and parasitologically in one of the cases (Neva, Petersen and Corsey of BEIB). Cultures of weighed skin biopsies and serially diluted suspensions revealed that the lesions of DCL patients contained 10^4 to 10^5 culturable organisma per mgm of tissue.

Antibody Response to Different Antigens from Schistosomes. Previous work on antigens extracted from adult schistosomes showed that there were two components of a TCA-soluble crude fraction to which antibodies could be demonstrated. One, a proteoglycan associated with gut epithelial cells of the worm (GASP), reacts with antibody only from relatively recent schistosome infections. Antibodies to the other antigen, a polydisperse glycoprotein (PSAP), were found to reflect intensity of infection as judged by egg excretion. ELISA tests were previously used to measure antibodies to both GASP and PSAP antigens, but now radio-immuno assays have been developed which are more sensitive and specific. It has been possible to estimate the amount of GASP antigen elaborated *in vitro* per worm per day as about 140 to 270 ng by the radio-immuno assay. The test has a sensitivity of 1 to 10 ng per ml, but even with this radio-immuno assay, antigen has not been found in the serum or urine of heavily infected patients (Nash).

Immune Response to Helminth Infections. The fact that high serum IgE levels are often seen with helminthic infections, particularly, would lead one to expect allergic symptoms as being much more frequent in such patients. A partial explanation for the relative infrequency of immediate hypersensitivity in parasitic infections is now available in the demonstration of blocking antibodies that inhibit histamine release in the serum of filariasis patients. The blocking activity was found to be due to a serum factor, presumably an antibody of the IgG class. The blocking antibody is antigen specific, since it does not block ragweed-induced or heterologous parasite-induced histamine release. Also, similar blocking antibody has been found in patients with schistosomiasis (Ottesen and Hussain). Another question raised by the high prevalence of parasitic worm infections in some parts of the world is whether there might be some immunologic consequences in the newborn of parasitic infections during pregnancy. One aspect of this issue was examined with regard to filariasis in India in 40 pairs of maternal and cord bloods that were checked for microfilariae and serum IgE levels. Eight of the 40 maternal bloods were positive for microfilariae, but none of cord bloods were positive. However, sera from 10 of the 40 cord bloods has IgE, presumably trans-placental in origin. Specificity of the IgE will be determined (Weil, Ottesen and Indian collaborators).

Since mass chemotherapy of schistosomiasis is being used increasingly in control programs, the effect of treatment on immunologic responsiveness seemed desirable to investigate. This is under study in a Puerto Rican population chronically infected with *S. mansoni* who have been treated with oxamniquine. A complete profile of cell-mediated, delayed and immediate hypersensitivity responses to several different schistosome antigens has already been done and will be repeated after treatment in selected patients (Hofstetter, Ottesen and Ruiz Tiben).

The recent translocation of Southeast Asian refugees to this country has resulted in the referral to us of new and unusual problems of parasitic infections. One such example is a new species of human schistosome infection, *S. mekongi*, which is similar to but distinct from *S. japonicum.* The first immunologic assessment of *S. mekongi* infections has been carried out in a family of Laotian refugees, with the finding that they show a similar pattern of cell-mediated responses as seen in patients infected with *S. mansoni* or *S. hematobium* (Barral and Ottesen). These patients with *S. mekongi* infection present a particular problem in regard to treatment since this infection is presumably resistant to the usual anti-schistosomal drugs, as are *S. japonicum* infections. Therefore, a special protocol has been arranged to treat the 11 or 12 available cases with a new drug, praziquantel, which has been found to be the most effective agent for oriental schistosomiasis in trials outside of the U.S.A. (Nash and Ottesen).

Publications:

1. Nash, T.E.: Schistosoma mansoni: Pattern of release of secretory products. Experimental Parasitology. 48: 92-99, 1979.

2. Ottesen, E.A.: Modulation of the host response in human schistosomiasis. I. Adherent Suppressor Cells that Inhibit Lymphocyte Proliferative Responses to Parasite Antigens. J. Immunol. 123: 1639-1644, 1979.

3. Ottesen, E.A.: Immunopathology of lymphatic filariasis in man. Springer Semin. Immunopathol. 2: 373-385, 1980.

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5. Ottesen, E.A.: Visceral larva migrans and other unusual helminth infections of man. In Principles and Practice of Infectious Diseases. G.L. Mandell, R.G. Douglas Jr., and J.E. Bennett, eds.: Wiley & Sons, New York, p. 2191-2198, 1979.

6. Ottesen, E.A. and Poindexter, R.W.: Modulation of the Host Response in Human Schistosomiasis. II. Humoral Factors Which Inhibit Lymphocyte Proliferation to Parasite Antigens. Am.J.Trop.Med.Hyg. 29: 592-597, 1980.

7. Catto, B.A. and Ottesen, E.A.: Serotonin uptake in schistosomules of Schistosoma mansoni. Comp. Biochem. Physiol. 63C: 235-242, 1979.

8. Lawley, T.J., Ottesen, E.A., Hiatt, R.A. and Gazze, L.A.: Circulating immune complexes in acute schistosomiasis. Clin. Exp. Immunol. 37: 221-227, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 A1 00048-10 LCI

PERIOD COVERED
September 30, 1979 to October 1, 1980

TITLE OF PROJECT (80 characters or less)

The Pathophysiology of Autoimmune Hemolytic Anemia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Other:	M. Hamburger	Clinical Associate	LCI	NIAID
	L. Fries	Clinical Associate	LCI	NIAID
	A. Fauci	Head, Clinical Physiology Section	LCI	NIAID

COOPERATING UNITS (if any) T. Lawley, R. Hall, S. Katz (Dermatology Branch,NCI/NIH); P. Plotz (Arthritis Branch, NIAMDD/NIH); H. Moutsopoulos, (NIDR); T. Chused, (NIAID); E. Franklin, (NYU School of Medicine); G. Sharp, (Univ. of Missouri School of Medicine).

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have continued to extend our new technique for measuring IgG Fc receptors in patients with autoimmune disease. These techniques have proven useful in diagnostic tests and for developing an understanding of pathophysiologic processes. Patients with mixed IgM, IgG cryoglobulins can be divided into two groups. The patients with Fc receptor defects are the ones with immune complex mediated renal disease. The patients with normal Fc receptor function do not appear to develop immune complex mediated renal disease. The follow-up studies have performed in our patients with lupus erythematosus. As the patients improve the immune clearance defect also improves. Patients with mixed connective tissue disease have been studied and have been shown in general to be free of defects in immune clearance not withstanding the fact that they have circulating immune complexes. Current studies demonstrate an Fc receptor defect in patients with Dermatitis Herpetiformis. Because these patients are all HLA B8 Dw27 we have extended our studies to normal HLA B8 Dw27 individuals. 50% of these have an Fc defect suggesting that this may be one cause of autoimmune disease.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To define the pathophysiologic function of antibody and complement on the surface of red cells in patients with hemolytic anemia, and to use the findings of this study to develop new tests for immunologically specific, reticuloendothelial system macrophage membrane receptors in patients with a variety of disease states. The tests developed for the study of autoimmune hemolytic anemia have been found to have wide application to other autoimmune and immunologic problems.

Methods Employed:

These studies examine the clearance of radiochromated erythrocytes from the circulation. The initial studies were performed in a guinea pig model. The recent studies have been performed in human volunteers or in patients with various diseases. In the intial studies in humans, the clearance of erythrocytes coated with IgM isoagglutinin antibodies with or lacking membrane bound complement was examined. In later studies, radiochromated erythrocytes were coated with highly purified human IgG anti-Rh antibodies and the survival of these cells was also studied. The third general set of clearance studies examined the clearance of radioactive aggregated albumin. This is the standard method for determining RES clearance and can be used to determine both liver blood flow and the ability of the reticuloendothelial system to clear particulate materials.

Major Findings:

In past years, we identified the immunologically active protein fragments which, when deposited on red cells, are responsible for the clearance of those cells from the circulation. It was reported that receptors for these immunologically specific fragments were present on the membrane of macrophages and the receptors were responsible for removal of cells coated with these fragments from the circulation. It became clear that it was possible to study patients with a wide variety of diseases to determine whether their receptors for these immunologically active fragments were functioning normally *in vivo*. It has been known for years that the reticuloendothelial system (RES) plays a major role in removing foreign material from the circulation. These foreign materials include bacteria and parasites, as well as endogenously generated immunoreactive materials such as immune complexes. It was suspected that defects in RES function might be important in both infectious and immunologic diseases. However, when this question was examined directly using the techniques available, no defects in RES function were noted in any disease state in which blood flow to the RES organs was normal. Until the present studies, the technique available for examination of

RES function was that of study of clearance of aggregated albumin from the circulation. This study has led to the development of new techniques for evaluation of RES function. Using these new techniques developed within our unit, we have shown that there are indeed diseases with defects in RES function. Thus, receptors for complement and immunoglobulin on a patient's macrophages will recognize these immunologically active materials coating foreign substances as they circulate.

The defects are quite specific for different sets of receptors. Our test utilizes the patient's own red blood cells. The cells are coated with antibody and/or complement fragments and are infused into the patient and their clearance is followed. Using this technique, we have found that almost every patient with active lupus erythematosus has a major defect in Fc receptor specific clearance, although the clearance of aggregated materials is normal. We believe that it is quite likely that this clearance defect extends to the clearance of the circulating immune complexes which occur in these patients. If such complexes are not cleared efficiently from the circulation, they can be deposited in glomeruli where they can cause immune damage. We find that as the disease improves, the clearance defect also tends to improve and the complexes present in the circulation tend to disappear. However, it is striking that most patients with lupus erythematosus have a persistence of their clearance defect for long periods of time, even on treatment. Our studies in lupus erythematosus have already been confirmed by a group in London. These initial findings in lupus were published recently in the New England Journal of Medicine.

Extensive studies have been conducted of Sjögren's Syndrome. These studies demonstrated that patients with clearance defects and circulating immune complexes tend to develop peripheral manifestations of their Sjögren's Syndrome and extensive tissue injury. On the other hand, patients who do not have clearance defects tend not to develop secondary manifestations of their Sjögren's Syndrome with peripheral tissue destruction, even in the presence of circulating immune complexes. This material has recently appeared in the Annals of Internal Medicine. We have now had an opportunity to analyze the circulating immune complexes in patients with Sjögren's Syndrome, and we have shown that a very high proportion of patients have immune complexes and that these complexes are of multiple types. Moreover, the rheumatoid factor present in these patients contributes to only one type of complex and is not responsible for many of the positive tests for immune complexes noted in the serum of these patients.

With Dr. Edward Franklin and his group at NYU, we have had an opportunity to study patients with IgM-IgG mixed cryoglobulinemia. These patients have been shown to fall into two groups with regard to the development of peripheral manifestation of disease, in spite of the fact that all have circulating immune complexes. One group of patients has been shown to possess normal Fc receptor function, and these patients do not develop certain severe peripheral manifestations of disease such as immune complex mediated glomerulonephritis. A second group of patients have been shown to have a clearance defect, and it is these patients that have evidence of glomerulonephritis. Once again, the usefulness of the clearance studies in determining the nature of the pathophysiologic process in this disease is clear.

We have also studied a large group of patients with mixed connective tissue disease with Drs. Gordon Sharp and Harry Moutsopoulos. These patients tend not to have tissue deposition of immune complexes and extensive tissue damage, although they have circulating immune complexes in quantity. Interestingly, our studies show little or no evidence of a clearance defect in these patients except for those who have many of the manifestations of systemic lupus erythematosus. The latter patients tend to resemble patients with lupus erythematosus in that they have anti-DNA etc., and interestingly, these patients do have clearance defects. Finally, we have studied a group of patients with rheumatoid arthritis. These patients do not have striking Fc receptor clearance defects suggesting that much of the immunopathology that occurs is not systemic immune complex mediated injury but originates in the joints themselves.

Most recently we studied patients with Dermatitis Herpetiformis in collaboration with Drs. Lawley, Hall and Katz of the Dermatology Branch, NCI. It was found that 50% of these patients have a clearance defect. Because these patients have a particular HLA type, B8 DW 27, we studied normal volunteers with this haplotype. 50% of these normal individuals had an Fc receptor defect. Moreover these patients were shown by Dr. Fauci to have a defect in T lymphocyte Fc receptor expression. Thus, the defect in Fc receptors may precede the development of disease. This study provides the first link between HLA type and autoimmune disease and may prove a critical link between the known association of certain HLA types and autoimmunity.

Significance to Biomedical Research:

These techniques allow for the complete reevaluation of reticuloendothelial system function in man. Already they are being widely applied. The use of these techniques has already allowed for the description of the first patients with receptor specific defects. C3b specific defects have been demonstrated in primary biliary cirrhosis and Fc receptor defects in a number of serious autoimmune diseases. These techniques provide a powerful new tool in the evaluation of these patient groups and provide a new approach to understanding the pathophysiologic basis of these diseases.

Proposed Course:

We intend to extend these studies to additional autoimmune diseases. Moreover, we intend to attempt to determine whether patients with various kinds of infectious disease may also have clearance defects which contribute to their inability to clear bacteria from the circulation.

Publications:

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2. Lawley, T., Moutsopoulos, H.M., Katz, S.I., Theofilopoulos, A.N., Chused, T.M., and Frank, M.M.: Circulating Immune Complexes in Sjögren's Syndrome. J. of Immunol. 123: 1382-1387, 1979.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 AI 00049-09 LCI

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Initiation and Regulation of Antigen Recognition

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	K.U. Cehrs	Bio. Lab. Tech. (Micro.)	LCI, NIAID
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	J. Schroer	Guest Worker	LCI, NIAID
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COOPERATING UNITS (if any)

NONE

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SECTION
Biologic Structure Section

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NIAID/NIH Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5 11/12	4 5/12	1 6/12

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

INACTIVE DURING CURRENT YEAR. TERMINATED

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00050-10 LCI

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Clinical Studies of Complement Abnormalities of Man

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Frank	Head, Clinical Immunology Section and Chief, LCI	LCI	NIAID
OTHER:	S. Hosea	Senior Investigator	LCI	NIAID
	K. Katusha	Microbiologist	LCI	NIAID
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COOPERATING UNITS (if any)
D.W. Alling, OSD, NIAID, NIH: D. Triantaphylapolis and M. Wickerhauser
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 11/12	11/12	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

New therapies have been developed for treatment of hereditary angioedema, a disorder of the complement system with a previously high mortality. We have developed, with the Red Cross, a purified C1 esterase inhibitor preparation that reverses the blood biochemical abnormality and, also, ends acute attacks of disease. We have developed chronic therapy which leads to clinical cure of this disease, and often, causes complete correction of the biochemical abnormality in this genetically controlled deficiency state. Analysis of the mechanism of correction has led to a unified genetic hypothesis for the origin of the disease. We have provided definitive data on the incidence of drug toxicity in this disease and showed that danazol predisposes certain patients to develop insulin resistant diabetes.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

The general objective of this program has been to define the role of complement in human illness and to develop better methods of therapy for immune damage mediated by complement. Major emphasis has been placed on our studies with patients with hereditary angioedema. This illness, which carries a high mortality rate, is particularly useful for study since it can serve as a model for complement-mediated immune damage in man and as model for the study of genetically controlled autosomal dominant diseases. We have been particularly interested in defining the usefulness and toxicity of danazol in a large patient population. We are also interested in extending our efforts to stop attacks of hereditary angioedema once they have begun. There is, at present, no effective therapy for acute hereditary angioedema attacks.

Methods Employed:

Purified complement components are prepared, and antisera to various complement components and component inhibitors are used for estimation of component levels in human sera and other body fluids. Double-blind therapeutic studies are employed to test new methods of therapy. Patients on experimental drug protocols are followed to determine signs of toxicity.

Major Findings:

In the past several years, we have developed and evaluated a new drug, danazol, for treatment of hereditary angioedema. Earlier, we showed that this drug is markedly effective in treating this disease and is relatively non-toxic. The drug has the striking effect of causing patients with the deficient serum inhibitor protein to synthesize this protein so that levels of the protein in blood return to normal. With the restoration of normal levels of the protein in blood, the patient corrects his biochemical abnormalities and, thereafter, is free of symptoms. The long-term use of this new drug has never been studied in any patient population, and it was essential to begin to define those situations in which the drug was useful or, on the other hand, was likely to cause problems. We also were interested in attempting to stop attacks of hereditary angioedema once they had begun. It takes days for danazol to have its effect. Patients who are not on danazol, however, will often present to an emergency ward having severe angioedema which may be life-threatening. At the present time, there is no safe method of treating such patients. The use of fresh, frozen plasma has been advocated, since fresh plasma has the protein which the patients are missing; however, fresh plasma also has complement components in it, and the patients are depleted of these components at the time of an attack. It has been suggested that the infusion of complement components will make these patients much more ill before they improve. Thus, fresh plasma is not advocated for use in this disease.

We now have 63 patients on danazol therapy. We have developed considerable further experience with danazol and are now the leading group in developing methods to evaluate danazol toxicity. We are also now the largest group following patients with HAE on therapy and have begun to pull together our studies of the incidence of autoimmune disease in this interesting disease. We now have several patients with autoimmune thyroiditis, glomerulonephritis, regional enteritis, ulcerative colitis, pancreatitis, etc.

Our infusion studies are also progressing in a highly satisfactory way. We have assisted the Red Cross in preparation and in evaluation of C1 esterase inhibitor. Moreover, we have now completed a series of infusions of purified C1 esterase inhibitor into patients with HAE at times when they were not experiencing attacks, and also, have infused the protein into patients in the midst of attacks. These studies have shown that infusion of C1 esterase inhibitor is quite benign and does not carry the risk of hepatitis. When infused into patients free of clinical symptoms, these patients experience an elevation of C1 esterase inhibitor and a subsequent elevation of C4 levels, as might be expected from pathophysiologic mechanism of this disease process. When infused into patients experiencing attacks of HAE, attacks have been aborted in as short a period as 5 minutes and as long a period as several hours. It would appear that this will afford an excellent approach in emergency rooms for treating patients with lifethreatening attacks of hereditary angioedema.

We are continuing to extend our understanding of Hereditary Angioedema treatment. This year we began to evaluate patients for signs of myopathy and have shown that a unique myopathy develops in patients with this disease. Moreover, we showed that danazol predisposes some patients to develop insulin resistant diabetes which at times can itself be highly toxic due to the development of profound hypoglycemia.

We are also conducting ongoing studies of the genetic defect in hereditary angioedema. Two types of patients have been described. The majority of patients have low levels of a normally functioning protein. A small percentage of patients have normal or elevated levels of a non-functioning gene product. Our studies suggest that patients with all of the various phenotypic varients of hereditary angioedema are heterozygous for the gene defect. All patients appear to have the ability to synthesize the normal gene product when they are appropriately stimulated. Danazol appears to be the best drug in providing this appropriate stimulation. In the common disease varient, the patients appear to have one silent gene. In the abnormal varient form, the abnormal gene directs synthesis of a non-functioning gene product. Thus, we have advanced a unified hypothesis for the genetic basis for this biochemically pleomorphic disease.

Significance to Medical Research:

Our laboratory has been the focus for most of the recent therapeutic developments in current treatment of this disease. Patients with hereditary angioedema experience a 25% mortality and have extensive morbidity from their constant attacks of the disease. As a result of our therapeutic interventions these patients lead a normal life. Moreover, our development of drugs and our study of their mechanism of action has led to a far greater understanding of the genetic basis of the protein abnormality underlying the disease. At present, this disease represents one of the few genetically controlled disorders in which there has been biochemical correction and therapy cure of a severe illness.

Proposed Course:

We are continuing to evaluate the long term use of drugs in patients with this particular disease. We are also gradually increasing the number of other patients with other complement-related abnormalities which are under study. These include such diverse illnesses as autoimmune hemolytic anemia and systemic lupus erythematosus.

Publications:

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10. Hosea, S.W., Santaella, M.L., Brown, E.J., Berger, M., and Frank, M.M.: Long-term therapy of hereditary angioedema with danazol. Ann. Intern. Med. In press.

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1. Gelfand, J.A., Rosa, G.R., Conley, C.L., Allen, J.C., Reinhart, R., Humphrey, R.L. and Frank, M.M.: Acquired Cl Esterase Inhibitor Deficiency and Angioedema. Medicine 58: 321-28, 1979.

2. Atkinson, J.P. and Frank, M.M.: The Complement System. In Parker, C. (Ed.): Clinical Immunol. Philadelphia, PA, W.B. Saunders Co. 1980, 219-271 pp.

3. Parillo, J.E., Lawley, T.J., Frank, M.M., Kaplan, A.P. and Fauci, A.S.: Immunologic Reactivity in the Hypereosinophilic Syndrome. J. Allergy Clin. Immunol. 64:113-121, 1979.

4. Frank, M.M.: The Effect of Sex Hormones on a Complement Related Clinical Disorder, Hereditary Angioedema. Proceedings of the Kroc Foundation Meeting on Sex Factors, Steroid Hormones and the Host Response. Amacher, P. and Talal, N. (Eds). Arthritis Rheum. 22: 1295-1299, 1979.

5. Gadek, J.E., Hosea, S.W., Gelfand, J.A. and Frank, M.M.: Response of Variant Hereditary Angioedema Phenotypes to Danazol Therapy: Genetic Implications. J. Clin. Invest. 64: 280-286 July, 1979.

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8. Hosea, S.W., Santaella, M.L., Brown, E.J., Berger, M., and Frank, M.M.: Long-term therapy of hereditary angioedema with danazol. Ann. Intern. Med. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00055-08 LCI

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Regulation of the Immune Response in Man

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A.S. Fauci	Head, Clinical Physiology Section	LCI NIAID
		Deputy Clinical Director	NIAID
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	T. Cupps	Clinical Associate	LCI NIAID
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	B. Haynes	Senior Investigator	LCI NIAID
	P. Katz	Clinical Associate	LCI NIAID
	C. Lane	Clinical Associate	LCI NIAID
	H. Stevenson	Clinical Associate	LCI NIAID
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	G. Whalen	Biologist	LCI NIAID

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Clinical Investigation

SECTION
Clinical Physiology Section

INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6 5/12	4 5/12	2

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The precise mechanisms of triggering of human B lymphocytes as well as the immunoregulatory events associated with non-specific and antigen specific stimulation have been delineated in normal individuals and abnormalities of cell triggering and immunoregulation have been described in several immunologically mediated diseases. Hybridoma monoclonal antibodies against human mononuclear cell subsets have been produced and these have been successfuly employed to precisely delineate immunoregulatory lymphocyte subsets in normals and disease states; they have also been employed to characterize the ontogeny of functional T cell subsets as well as the differentiation levels of malignant transformation of certain lymphoproliferative diseases. The first antigen specific human T cell clones have been established; they are true clones, totally antigen specific and strictly genetically restricted regarding the requirement for antigen presenting cells. Modulation of the functions of lymphocyte subsets by several relevant pharmacologic agents have been defined. Clinical pathophysiologic and therapeutic studies have been performed in the systemic necrotizing vasculitides and extraordinarily favorable results with the use of cytotoxic agents have been reported.

Project Description

Objectives:

1) To delineate the mechanisms of regulation of immunologic reactivity by purified subpopulations of human lymphoid cells. To examine the precise functional capabilities of these individual lymphoid cell subpopulations as well as the mechanisms whereby they maintain the normal homeostatis of positive and negative signals which regulate normal immunologic reactivity.

2) To determine the role of these lymphoid cell subpopulations in the pathogenesis of immunologically mediated diseases such as the vasculitides, hypersensitivity diseases, granulomatous diseases, the spectrum of autoimmune diseases, as well as infectious diseases characterized by aberrations of immunologic reactivity. In particular, to delineate the alterations of various subpopulations of cells by mechanisms such as a) modulation of cell surface receptors (for example, Fc receptors) by immune complexes, cryoglobulins or other serum factors; b) activation of cell subsets by viruses (such as Epstein-Barr virus); and c) depletion, proliferation, or redistribution of cell subsets among lymphoid compartments. In this regard, to determine the role of these mechanisms in the abnormalities of immunoregulation characteristic of these diseases.

3) To develop hybridoma cell lines which secrete monoclonal antibodies directed against cell surface antigens on human lymphocyte and monocyte subpopulations, and to employ these monoclonal antibodies to define distinct subpopulations of immunoregulatory lymphoid cell subsets and effector cell subsets involved in normal human immunologic reactivity. In addition, to precisely delineate lymphoid cell lineage and ontogeny and to define the ontogenic level of malignant transformation of lymphoid cell subsets based on the differential expression of these surface antigens. Furthermore, to define the role of mononuclear cell subsets expressing these surface antigens in the development and functional expression of autoimmune and immunodeficiency diseases.

4) To develop antigen specific human T cell lines derived from a single cell, hence antigen specific T cell clones. To define the culture conditions for the establishment of such clones and to characterize these cloned cells with regard to the expression of surface markers, functional capabilities (help, suppression, cytotoxicity, etc.), antigen specificity, reliance upon auxilliary cells for presentation of antigen, degree of genetic restriction in expression of antigen-specific responses. Of particular importance is the development of antigen specific clones in order to approach the critical task of identifying and characterizing the receptor for antigen on human T cells.

5) To determine the role and precise mechanisms of anti-idiotypic antibodies in the regulation of human B cell responses in normal immune reactivity and in diseases characterized by abnormalities of immunoregulation. To identify the expression of and receptors for idiotypic and antiidiotypic determinants on human lymphocyte subsets.

6) To investigate the cellular interactions, molecular, and biochemical events associated with triggering of various mononuclear cell subpopulations and to determine the relationship between selective triggering via various cell surface receptors and the kinetics of expression of various functional capabilities of the given cell populations. In addition, to describe and characterize the receptors for triggering via mitogens, T cell factors, and other signals on the surface of responder T and B cell subsets. To induce, purify and characterize soluble factors from purified subpopulations of human lymphocytes and monocytes which are involved in the regulation of immunologic reactivity.

7) To determine the role of feedback inhibition and enchancement by antibody of various classes and soluble immune complexes in human B cell triggering, differentiation, proliferation and antibody production and secretion.

8) To perfect the development of culture and assay systems for the *in vitro* induction of antigen specific responses by human lymphoid cells in order to delineate the role of presenting cells, antigen specific helper and suppressor cells, antigen specific T cell receptors, and Ia molecules in the expression of antigen specific *in vitro* responses in man. To define the role of synergy between non-specific (polyclonal) and specific (antigen) signals in the expression of antigen specific B cell responses in man. In this regard, to investigate the role of polyclonal activation in the development of autoreactive responses against self-antigenic determinants. Furthermore, to define more precisely the primary versus secondary role of altered immunoregulation in the development and perpetuation of autoimmune disease.

9) To delineate the mechanisms whereby recognition of self molecules (such as Ia-like determinants) plays a role in non-self antigenic recognition. In this regard, to determine the role of autoreactivity in the normal immunologic homeostasis in man and to characterize the aberrancies of this otherwise normal level of autoreactivity which are associated with pathologic autoimmunity and altered immune surveillance in certain neoplastic diseases, particularly those of the lymphoproliferative type.

10) To define the precise physiologic and biochemical mechanisms whereby cellular cytotoxicity is expressed. In particular to delineate the mechanisms of natural killer (NK) cell activity versus antibody dependent cellular cytotoxicity (ADCC) against panels of nucleated and non-nucleated targets. To use human diseases which manifest defective NK and ADCC activity such as the Chediak-Higashi syndrome and chronic granulomatous disease respectively as naturally occuring human models of selectively defective cytotoxic systems, and to employ these models to define the normal mechanisms of NK and ADCC activity.

11) To develop methodologies of fractionating purified populations of human peripheral blood monocytes and T cell subsets by techniques such as elutriation centrifugation in order to avoid pre-activation of the responsive populations which invariably occurs with positive selection methodologies. In this regard, to utilize these purified populations of cells to determine in a more precise manner the functional capabilities and cellular interactions in which these subsets of cells participate in immune reactivity.

12) To delineate the precise mechanisms whereby immunosuppressive agents such as corticosteroids and cytotoxic drugs affect the immune responses in man specifically examining their selective effects on subpopulations of lymphocytes and monocytes with regard to their circulatory kinetics, cellular interactions, activation, and expression of in vivo and in vitro functional capabilities. In addition, to correlate the selective effects of corticosteroids and cytotoxic drugs on these various parameters with the suppression of disease activity in various inflammatory and immunologically-mediated disease states in man, and by doing so, to aim at a greater understanding and therapeutic specificity in the clinical use of these agents. Furthermore, to study the mechanisms whereby manipulations of immune reactivity as described above affect host defenses against infection, tumor surveillance and propensity towards autoimmune states.

13) To investigate the multifacted aspects of eosinophil production, circulation, proliferation and effector function employing a large group of patients with the idiopathic hypereosinophilic syndrome (HES) as sources of material. Specifically, to define the aberrancies of stem cell proliferation and circulation in the HES as well as the role of mononuclear cell feeder cells in the regulation of eosinophil production. To define the complex mechanisms whereby eosinophils and/or their products damage selective organs such as the heart causing the classic lesion of HES, i.e. endomyocardial fibrosis. To identify the heterogenous stimuli to the relatively uninhibited proliferation of eosinophils in the HES. Using these approachs, to ultimately define the kinetics and effector function of eosinophils in the normal state.

14) To continue to study the broad spectrum of the Vasculitic Syndromes from a mechanistic, pathophysiologic, clinical and therapeutic standpoint. Specifically, to employ this large group of patients to dissect the mechanisms of altered immunologic reactivity which results in vasculitic syndromes. In addition, to continue to develop effective modalities of therapy for these serious systemic disorders in order to achieve maximal therapeutic efficacy with minimal toxic side effects of therapy.

Methods Employed:

1) The predominant theme of this laboratory is the precise delineation of the complex mechanisms of immunoregulation in normal human immunologic reactivity and in disease states characterized by abnormalities of immunoregulation and/or the expression of immune reactivity.

In this regard, a major component of the methodology relates to the fractionation, identification and purification of immunoregulatory lymphoid cell subpopulations and the in vitro culturing of these cells to define their diverse functional capabilities. Subpopulations of cells are identified by surface markers and functional capabilities are assessed by a variety of in vitro assays. We have focused on the relationship between various in vivo and in vitro triggering signals and the subsequent expression of direct and immunoregulatory functional capabilities. This has been focused predominantly in the area of activation, proliferation and differentiation of human B lymphocytes to antibody producing and secreting cells. In this regard, we have been utilizing a unique model of human B cell function which was originally developed in this laboratory. It is a system of primary in vitro stimulation of bone marrow derived (B) lymphocytes by polyclonal activation as well as specific antigenic stimulation with subsequent measurement of single cell antibody production by a direct hemolysis-in-gel plaque forming cell (PFC) assay. We have further extended this system to now measure indirect (IgG) PFC. We also have developed a system for the measurement of Ig secreting cells of all classes by use of staphylococcal protein A (SPA) coated target erythrocytes and class specific developing antisera.

In addition, highly sensitive assays (enzyme linked immunosorbent - ELISA) have been developed and are being employed to measure the in vitro production of specific antibody following in vivo immunization of human subjects as well as primary in vitro antigenic stimulation.

Other assays employed to delineate the functional capabilities of lymphoid cells are the in vitro blastogenic responses to mitogenic and antigenic stimulation, elaboration of various soluble mediators, cell mediated cytotoxicity against various autologous, allogeneic and xenogeneic targets. Various cytotoxicity assays include spontaneous, mitogen dependent, and antibody dependent cellular cytotoxicity. A particular aim is to identify and characterize the abnormalities of lymphocyte or monocyte subpopulations involved in immunologically mediated diseases to determine the primary or secondary nature of these abnormalities and their relationship to the altered state of immune reactivity, as well as to create in vitro models of altered immunoregulation by selectively triggering or manipulating immunoregulatory subpopulations of cells.

2) Lymphocyte subpopulation are identified by unstable cell surface markers such as Fc receptors for various classes of Ig. In addition, heteroantisera against functional lymphoid cell subsets were employed. Recently, we have developed a panel of hybridoma monoclonal antibodies against functionally defined lymphocyte subsets as well as against lymphocyte subsets whose precise functional capabilities have not as yet been clearly defined. The use of hybridoma antibodies against lymphocyte subsets has proven invaluable as methodologic tools in the precise delineation of lymphocyte subsets in normal and aberrant immunologic reactivity.

3) We have developed the first antigen specific human T cell clones and we are now employing this unique and revolutionary methodology to approach a number of questions such as the nature of the antigen receptor, the genetic restrictions in cellular interactions and the role of presenting cells in antigen specific human T cell responses.

4) Recently developed elutriation centrifugation techniques are being employed to isolate purified (greater than 95%) populations of human monocytes from peripheral blood, thus avoiding adherence techniques.

5) In studies involved in the delineation of antigen specificity of human B cell responses, normal volunteers are immunized with various antigens such as keyhole limpet hemocyanin (KLH) and tetanus toxid. Their lymphocytes are subsequently cultured as described above in order to determine the cellular interactions, genetic restrictions, role of presenting cells and role of specific and non-specific signals in the *in vitro* induction of antigen specific B cell responses. In addition, sera from immunized subjects are purified by affinity chromatography to obtain specific Ig of various classes in order that immune complexes may be made *in vitro*. These immune complexes are then employed in the *in vitro* culture systems to determine the role of specific IgM versus IgG antibody as well as immune complexes in various states of antigen or antibody excess in the induction and regulation of specific B cell responses.

6) Methods to fractionate purified populations of eosinophils (90-98% pure) from human blood have been developed and are being employed.

7) Corticosteroids are administered to normal volunteers and patients requiring this drug and selective effects of these agents on the compartmentalization and functional capabilities of monocytes and lymphocyte subpopulations are determined. In addition, the differential effects of *in vitro* corticosteroids and *in vitro* irradiation on various lymphoid cell functional capabilities such as triggering, proliferation, differentiation, antibody production and secretion are being studied. In patients receiving cytotoxic agents (cyclophosphamide, azathioprine, etc.) for the therapy of immunologically mediated diseases, studies are performed on lymphoid cells in the same manner as that mentioned above for corticosteroid administration to normal volunteers and patients.

8) Subjects' lymphoid cells are labeled with ^{51}chromium and reinfused back into their circulation to determine the effects of disease states and of chemotherapeutic agents on the viability and circulation patterns of these cells.

9) Bone marrow aspirates are performed in normal subjects and patients with various diseases prior to, during and following therapy. Purified populations of lymphoid cells are obtained from these bone marrow cell suspensions by various separation techniques in order to delineate the immunocompetence of bone marrow lymphoid cells in normal man, as well as the alteration of compartmentalization and function of these cells in untreated and treated disease states.

Major Findings and Significance to Biomedical Research and the Program of the Institute:

A number of major and significant findings in human immunobiologic research have emerged from this laboratory over the past year. These have related to several general areas such as immunoregulation in normal immune reactivity; immunoregulation in diseases characterized by abnormalities of immune reactivity; dissection of the mechanisms of NK and ADCC; development of several hybridoma cell lines secreting monoclonal antibodies against functionally defined mononuclear cell subsets; establishment of the first antigen specific human T cell clones; description of the first example of suppression of human B cell function by heterologous anti-idiotypic antibody; delineation of the role of diverse modulatory substances such as corticosteroids, cytotoxic agents, cyclic nucleotides, ascorbic acid and other agents in the regulation of human immune reactivity; delineation of the mechanisms of action of corticosteroids and cytotoxic agents on immune reactivity in disease states; establishing therapeutic modalities for various diseases, particularly the vasculitic syndromes and other immune mediated inflammatory diseases; and intensive investigations of the pathophysiologic mechanisms of the hypereosinophilic syndrome.

Specific findings of particular relevance include:

1) Mechanisms of Activation of Human B Lymphocytes. Major advances have been made in the delineation of the mechanisms of activation of human B lymphocytes. We had originally described unique and precise assays for the induction and measurement of human B cell function. These were applied in both the mitogen-induced and antigen specific systems. They include the first polyclonally induced as well as antigen specific direct and indirect hemolytic plaque forming cell (PFC) system for use with human B cells. To this approach we have added a highly sensitive enzyme linked immunosorbent assay (ELISA) to detect minute amounts of Ig and specific antibody produced in *in vitro* human lymphocyte cultures. Our systems have been now adapted by a number of laboratories throughout the country and the world. Employing these systems, we have been involved in delineating the complex mechanisms of human B cell triggering. We have described the synergistic interaction between non-specific (polyclonal) and antigen specific signals in the induction and expression of antigen-specific responses. These findings have important implications in our understanding of the role of polyclonal activation in various autoimmune diseases, particularly systemic lupus erythematosus (SLE).

In addition, we have precisely defined the role of the monocyte in the mitogen and antigen induced triggering of human B cells. In non-specific (mitogen) triggering of B cells, the monocyte serves as a focusing cell which actually "presents" the mitogen to the lymphocytes (T cell plus B cell). This is particularly true with the mitogen pokeweed mitogen (PWM); we have demonstrated that the monocyte has a receptor for PWM and the mitogen selectively binds to the monocyte. Most importantly, the mitogen is presented by the monocyte to the responding lymphocyte in association with monocyte derived Ia since treatment of the monocyte with anti-Ia after "pulsing" with PWM blocks the ability of the monocyte to serve as a presenting cell.

Furthermore, mitogenic factors can directly trigger B cells (probably in cooperation with helper T cells) to secrete Ig. Thus, the lymphocytes have receptors for the mitogenic factors. Monocytes on the other hand also have receptors for the mitogenic factor and can interfere with the triggering of lymphocytes by the factors by competing for the factors via their own receptors. Finally, we have demonstrated that one can induce specific IgG antibody production by IgM-antigen complexes.

Appreciation of the immunobiological mechanisms of B cell triggering has obvious important implications in our understanding of the mechanisms of disease activity in autoimmunity and immunodeficiency states. In addition, the demonstration of the induction of B cell reactivity by a combined recognition of self and non-self determinants provides a basis for experimental distinction between non-pathologic and pathologic autoimmunity.

2) Immunoregulation in Normal Immune Reactivity. Precise delineation of immunoregulatory lymphocyte subpopulations in normal invividuals has been accomplished. We have previously reported the original description of a delicate balance between helper and suppressor cell subpopulations in the modulation of normal B cell immunologic reactivity. In addition, we have demonstrated that certain otherwise normal individuals are hypo-responders in these in vitro B cell systems, and that this hyporesponsiveness resulted directly from overly active or triggered suppressor cells. We have now extended these studies to precisely characterize the identity and nature of this naturally occuring suppressor T cell.

In addition, we have characterized the precise identity, kinetics and mechanisms of action of the mitogen (Con-A) induced suppressor cell in normal humans.

Using the elutriator centrifugation technique developed in our laboratory, we have been able to fractionate highly purified populations of human monocytes from peripheral blood mononuclear cells by negative selection, hence without triggering the cells. Using these cells we have demonstrated that not only is the monocyte absolutely essential for the mitogen induced B cell reactivity, but the monocyte is capable of functioning as a potent suppressor of B cell function. Of particular interest was the demonstration of the fact that in the monocyte induced suppressor phenomenon there is a clearcut interaction between the monocyte and an irradiation sensitive, Fc_G receptor positive lymphocyte. This is the first demonstration of a monocyte-T cell interaction in the immunoregulation of normal human B cell reactivity. In addition, we have demonstrated that factors produced by activated T cells can trigger resting monocytes to suppress B cell function.

Description of the immunoregulatory T cell subsets as defined by monoclonal antibodies developed in this laboratory will be described below in the section concerned with monoclonal antibodies.

Regarding the concepts of self and non-self recognition and its relationship to immunoregulation, we have investigated the mechanisms of regulation of autologous and allogeneic reactivity in the models of the autologous and allogeneic mixed lymphocyte reactions (MLR). In addition, we have defined the relative stimulatory and responder capabilities of various lymphocyte and monocyte subpopulations in the MLR. The relationship between the autologous and allogeneic MLR and the development of suppressor and/or cytotoxic T cells was described. Furthermore, the modulation of the autologous and allogeneic MLR in normal subjects by adherent cells, prostaglandins, irradiation and in vitro and in vivo corticosteroids has been delineated.

3) Immunoregulation in Disease States. Applying the above-mentioned systems of immunoregulation we have characterized the primary and secondary immunoregulatory abnormalities in a number of immunologically mediated diseases.

We have demonstrated the presence of altered proportions and absolute numbers of immunoregulatory T cell subpopulations in patients with systemic lupus erythematosus (SLE). In addition, we have demonstrated a deficiency of suppressor cell function in SLE patients. In this regard, an in vitro model of pre-triggering suppressor cells by activation with immune complexes has been developed to support the hypothesis of immune complex associated alterations of immunoregulation in SLE and related syndromes. We have defined the spectrum of relative alterations of immunologic reactivity by demonstrating the presence of mild hyperreactivity of B cells associated with reversible alterations of immunoregulatory T cell subsets in Sjogren's syndrome (SS) without other associated connective tissue disorders; modest to severe B cell hyperreactivity with irreversible alterations of immunoregulatory T cell subsets in SS associated with mild SLE; and severe B cell hyperreactivity together with alterations and depletions of immunoregulatory T cell subsets in frank SLE.

Of major importance is the fact that we have developed an anti-idiotypic antibody against the surface IgM and serum monoclonal IgM peak of a patient with chronic lymphocytic leukemia whose leukemic B cell clone is secreting monoclonal antibody against SRBC determinants. The anti-idiotypic antibody reacts with the patient's B cell surface IgM and serum IgM but not with normal B cell surface IgM or serum IgM. Furthermore, the anti-idiotypic antibody blocks the spontaneous and mitogen induced secretion of anti-SRBC antibody by the patient's B cells but not by normal B cells. This is the first demonstration in man of the inhibition of B cell function by anti-idiotypic antibody and has obvious important implications in the understanding of the role of the idiotype network in the regulation of human immunologic reactivity.

We have demonstrated the presence of spontaneously activated B cells in Epstein-Barr virus (EBV)-induced acute infectious mononucleosis (IM). We have further shown that this is due to the direct triggering of B cells by EBV via surface receptors. We have characterized the atypical lymphocyte in IM and have demonstrated for the first time the emergence of suppressor T cells of antibody production and B cell outgrowth in acute IM and their subsequent disappearance in the convalescent state. This has important implications as a potential mechanism of containment of disease activity in IM and prevention of evolution into a B cell neoplasm.

In addition, we have demonstrated the alteration of immunoregulatory T cell subsets as well as the presence of adherent suppressor monocytes in sarcoidosis and tuberculosis.

An extremely exciting development in the laboratory during the past year was the demonstration that patients with the Chediak-Higashi syndrome have an isolated deficiency of NK cells. This is the first description in man of a primary immunodeficiency disease with an isolated defect in NK cells. This was shown to be truly a functional defect since the abnormality was not due to a lack of effector cells or an inability of effector cells to bind normally to target cells. Furthermore, the defect was partially, but not completely corrected with interferon (*in vitro*). In addition, the defect was corrected by the addition of cyclic nucleotides (cyclic GMP) to cultures. The details and significance of these findings will be discussed below. In any event, the isolated NK defect in the Chediak-Higashi syndrome might well explain the predisposition of these patients to develop lymphoproliferative malignancies.

4) Hybridoma Monoclonal Antibodies. In order to more precisely delineate the immunoregulatory lymphocyte subsets involved in the regulation of human lymphocyte (particularly B cell) function, we developed a panel of mouse hybridoma monoclonal antibodies against human mononuclear cell subsets. This proved to be extremely rewarding from several standpoints.

We described in detail adaptations and modifications of the original Milstein and Kohler methodology for hybrids. In these studies we described revised screening strategies which allowed us to be successful in establishing a number of hybridomas. We went on to describe several (five) in some detail.

The hybridoma which we have studied in most detail up until now is the 3A1 antibody. It reacts with approximately 85% of peripheral blood T cells and is an IgG_1 k. We described the blastogenesis profiles in which 3A1 positive T cells respond suboptimally to PHA and only minimally to Con A. Clearly most of the classic "T_G" cells are 3A1 negative; however, it is also clear that a percent of T_G cells (E rosette positive, Fc_G receptor positive) are indeed 3A1 positive. This establishes along with other data from this laboratory that the T_G cell is a heterogeneous population of cells which includes T cells and most likely monocytoid cells as suggested by data from other laboratories. We further went on to describe that the 3A1 positive T cells were the helper cells in mitogen induced B cell function. In addition, the Con A generated suppressor cells were contained in the 3A1 positive population, whereas the "spontaneous" suppressor cells were contained in the 3A1 negative population which was predominantly T_G. These findings have been of major importance in clarifying the controversy regarding the difference between inducible and spontaneously occurring suppressor cells. In addition, we demonstrated that the 3A1 positive cells did not mediate ADCC while the 3A1 negative cells did.

Of particular excitement was the finding that the Sezary T cell is 3A1 negative while still maintaining its functionally mature helper capabilities. This suggests that the Sezary cell lost its 3A1 antigen during malignant transformation and so exemplifies a clearcut dichotomy between a surface marker which represents a population of normal helper cells and the expression of this helper capability by malignant cells which do not express this antigen. Such findings have potentially extremely important implications in our understanding of the molecular events at the cell surface level which are associated with malignant transformation of lymphoid cells.

In this regard, we were fortunate enough to have come upon a patient with what appeared to be a "malignant proliferation" of T_G cells. Given the disagreements in the literature regarding whether what investigators have been calling T_G cells are indeed T cells, we studied this patient in detail. In one complete study, we have demonstrated that this patient had a proliferation of normal appearing and functioning cells which were low affinity E rosette positive and Fc_G receptor positive, hence a classic T_G cell. However, most importantly, these cells were 3A1 positive and so clearly were T cells. Furthermore, they suppressed normal human B cell function and functioned as normal NK cells. This study has definitively answered several questions of current great interest in human immunobiology:

the T_G cell is indeed a T cell; the T_G cell is the suppressor cell in spontaneous suppression of B cell function; and finally, this cell is also the same cell that mediates NK activity. Of great importance also was the finding that this cell elaborates interferon and the patient has high levels of circulating interferon.

Another antibody which we have described is the 4F2. It is an antibody which binds to monocytes and not to resting peripheral blood lymphocytes or even lymphoid organ lymphocytes. However, upon activation of lymphocytes, the 4F2 antigen is expressed. The antigen is not Fc receptor, not Ia and not HLA. Thus, there is antigen which is normally found on resting monocytes, but only found on activated lymphocytes. This finding has important implications in our understanding of the relationship between monocyte and lymphocyte lineage and also may serve as a unique marker of lymphocyte activation exclusive of Ia and Fc receptors.

We have also partially described the 5E9 antibody. It appears to be leukemic specific in that it binds to all acute lymphoblastic leukemia (ALL) T cell lines tested, it binds to certain EB virus transformed B cell lines, to Burkitt's lymphoma cells, acute myeloblastic leukemia (AML) cells, 30% of null cell ALLs and to some T cell ALLs. Yet, it does not bind to normal thymus, spleen, peripheral blood cells or to normal continuous T cell (CTC) lines. Thus, it is leukemic specific, but not leukemic type-specific.

Other antibodies which we are investigating from our panel is 3F10 which is an anti-HLA antibody similar to Milstein's W632. 3A6 is similar to 3F10 except that 3A6 blocks the binding of T cell growth factor (TCGF) to T cells whereas 3F10 does not. 5A12 antibody is essentially the same as 3A1 except that 3A1 is an IgG_1, k, while 5A12 is an IgM, k.

Thus the development and use of hybridoma monoclonal antibodies in this laboratory has proven to be extremely useful in delineating immunoregulatory mononuclear cell subsets as well as defining ontogenic and differentiation markers of normal and malignantly transformed cells.

5) Human Antigen Specific T Cell Clones. Adapting the methodology of R. Schwartz and B. Sredni who developed antigen specific T cell clones in the mouse model, we have successfully produced the first antigen-specific T cell clones in man and have perpetuated these with several sub-clonings. They are truly clones by the strict definition and have thus far been demonstrated to be antigen specific, genetically restricted by HLA and DR with regard to the absolute requirement for presenting cells, and are not alloreactive. The implications of developing antigen specific T cell clones are enormous. In addition to providing truly "pure" T cell populations for functional assays, it provides a source of T cell determinants without contamination from other cell types and other T cell subsets.

Thus, the pressing question of the nature of the T cell receptor can now be approached as well as other questions of the relationship between the expression of surface antigens and functional capabilities. In addition, since these cells are fully characterized with regard to HLA and DR, a whole host of studies relating to the genetics of human immune reactivity can be approached from an entirely new and potentially productive vantage point.

6) Technical Advance in Fractionation and Purification in Human Monocytes. We have adapted a unique cell fractionation technque called elutriation (counter-current centrifugation) for the purpose of purifying human blood monocytes. This technique provides a major advance in our ability to carefully and precisely study the human monocyte.

This technique has the advantage over the current adherence techniques of monocyte isolation of negatively selecting the human monocyte in excellent purity (95%) and excellent yield (95%). The yield of this procedure is so great, that up to 1.5 billion monocytes have been purified from the blood of a single normal human at one time. In addition, this technique has proved to provide much more effective monocyte depletion of mononuclear cell suspensions than the current technique of depleting adherent cells on sephadex G-10 columns since elutriation does not remove non-monocyte adherent cells (such as B cells). Thus, we have been able to characterize large numbers of purified human monocytes with regard to their intracytoplasmic enzyme activity. We have also examined monocytes with scanning and transmission electron microscopy for the details of their cytoskeleton assembly. In addition, we have placed large numbers of these cells into culture to examine how these parameters are altered when these cells mature into macrophages. We have been able to examine the evolution of these functions on a daily basis for one week using a single normal individual's monocytes. Human monocytes have been examined for their ability to perform the interrelated functions of phagocytosis and killing in an ADCC system. We have shown that while monocytes have improved phagocytic function after being placed in culture, they have diminished killing capability in ADCC. Of note is that we are the first group to demonstrate spontaneous killing by monocytes of human red cells and that this function improves when monocytes mature into macrophages. Hence, for the first time, human monocytes in a highly purified, non-activated state have been precisely characterized.

7) Delineation of the Mechanisms of NK and ADCC and Clinical Implications. As mentioned above, we have described the first primary immunodeficiency disease in man with a selective defect in NK activity in 2 patients with the Chediak-Higashi syndrome. These patients' monocytes and PMNs had normal

cytotoxic capabilities; however, their non-adherent mononuclear cells were markedly deficient in NK and to a lesser extent ADCC activity. The defect was partially, but not completely corrected by *in vitro* interferon. However, of great importance was the finding that cGMP or inducers of cGMP in culture could normalize the defective NK activity of the patients. Furthermore, pre-incubation of effector cells with cGMP with subsequent washing away of the compound also resulted in normalization of the NK effector function. Based on these studies, we treated the patients themselves with ascorbic acid (a known induced of intracellular cGMP) and tested the effect on their *in vitro* NK activity. While receiving ascorbic acid, the patients manifested NK activity which was almost normal, a marked improvement from pre-treatment levels. Of note was the fact that 48 hours after discontinuation of the drug, the NK activity reverted back to being markedly abnormal. These studies have extraordinary implications in the potential use of ascorbic acid therapy in the correction of certain NK defects.

In other studies, we demonstrated that patients with chronic granulomatous disease (CGD) have defective ADCC against non-nucleated target cells in cytotoxicity assays performed in the standard suspension cultures. In assays using human red blood cell (HRBC) targets, CGD patients had markedly abnormal killing by their PMNs and monocytes. Since this killing is associated with phagocytosis, the defect was felt to be due to a deficiency of a post phagocytic event since the targets were phagocytosed normally. Since CGD is a natural model of a deficiency of the post-phagocytic burst of oxidative metabolism, studies were performed on immobilized (adhered to glass plates) targets to prevent phagocytosis. Under these circumstances, CGD phagocytes killed non-nucleated targets extracellularly in a normal fashion. Furthermore, when lymphoid targets which are also not phagocytosed in cytotoxic assays were used, CGD effectors killed normally. Thus, the defect in CGD killing was related directly to a post phagocytic event. Since their classic intracellular microbial killing defect is a lack of the normal post-phagocytic burst of oxidative metabolism, clearly this can also explain the observed defect in this type of cytotoxicity. In addition to clarifying our understanding of the mechanisms of ADCC and the defect in this function in CGD, these studies also demonstrate for the first time that cytotoxic assays for ADCC as they are almost uniformly carried out really do not measure true extracellular killing, but are a reflection of a post-phagocytic process.

8) Pharmacologic Modulation of Immune Reactivity in Man. In line with our interest in the pharmacologic modulation of the immunoregulatory apparatus in man, we have studied and characterized the selective and differential effects of a number of agents directly on effector lymphocyte subsets as well as on functionally distinct immunoregulatory lymphocyte subsets. These agents include corticosteroids, cyclophosphamide, azathioprine,

irradiation, cyclic nucleotides, and prostaglandin inhibitors. A graded differential sensitivity of B cells, more than suppressor T cells, more than helper T cells was demonstrated to cyclophosphamide, azathioprine and irradiation. Cyclic AMP directly inhibited suppressor T cells, while prostaglandin inhibitors blocked prostaglandin mediated suppression of certain subsets of adherent suppressor cells. Corticosteroids had the most complex effects with selective and differential effects on the circulatory kinetics and functional capabilities of T and B cell subsets.

We have also delineated the relationship of corticosteroid receptors on lymphocyte subsets (with regard to receptor density, binding affinity and dissociation constant) and the differential effects of in vivo and in vitro corticosteroids on these subsets.

Of particular interest over the past year have been a series of studies aimed at determining the mechanisms whereby corticosteroids and cyclophosphamide successfully suppress the aberrant immunologic reactivity in our patients with immune mediated disease. In an extensive ongoing prospective study, we are determining the effect of these agents on the functional capabilities of immunocompetent lymphocyte subsets in patients prior to, during, and following discontinuation of therapy. This is the first study of this sort employing the currently available sophisticated immunologic technology. We had originally reported that patients with diseases such as SLE and systemic vasculitis have pre-triggered and activated circulating B cells secreting polyclonal antibody. Cyclophosphamide therapy results in a marked suppression of spontaneous and induced B cell function which appears to result from a direct suppression of the B cell itself since helper T cell activity remains relatively intact. Azathioprine appears to be far less suppressive of this B cell function. This may explain the consistent clinical observation that cyclophosphamide therapy is significantly more efficacious in the treatment of diseases characterized by hyperactive B cell function than is azathioprine.

In addition, the mechanisms of the selective effects of corticosteroids on lymphocyte subsets which we had previously delineated in normal individuals are now being confirmed in the patient populations.

Of particular interest regarding pharmacologic modulation of human immune reactivity are ongoing studies aimed at determining the role of interferon in immunoregulation of human B cell function. We have demonstrated that interferon has multifaced effects on human B cell function. At low concentrations it stimulates Ig secretion by B cells, but it does not have detectable inherent polyclonal B cell activating properties. Therefore, it is likely that it expresses this capability via an intermediary cell. Also, at higher concentrations, it suppresses B cell function by a mechanism that is not cytotoxic to B cells. The implications of delineating the role of interferon in immunoregulation are far reaching.

9) Clinically Related Studies. We have continued our clinical studies of a number of diseases of established or suspected immunologic mediation. These comprise virtually the entire spectrum of vasculitis including Wegener's granulomatosis, systemic necrotizing vasculitis (polyarteritis nodosa type), hypersensitivity vasculitis, Takayasu's arteritis, temporal arteritis, and lymphomatoid granulomatosis. In addition, we are studying idiopathic midline granuloma, sarcoidosis, granulomatous hepatitis, juvenile rheumatoid arthritis, Weber-Christian panniculitis, a heterogenous group of acquired immunodeficiency diseases, and host defense defects (chronic granulomatous disease, Chediak-Higashi syndrome), and a large number of patients with the idiopathic hypereosinophilic syndrome. The above patient groups are under our primary care. in addition, we are carrying on collaborative studies with others in SLE, Sjogren's syndrome, mixed connective tissue disease, rheumatoid arthritis, chronic lymphocytic leukemia, acute infectious mononucleosis, tuberculosis, and Cogan's syndrome.

In addition to carrying on extensive investigations delineating the mechanisms of altered immunologic reactivity in these diseases, the results of which are described above, we have established treatment protocols for several of these disorders. Major contributions have resulted from these and dramatic long-term remissions and even cures have been established by us in several of these formerly fatal diseases by the use of chronic low dose cytotoxic agents (usually cyclophosphamide), particularly in the severe systemic necrotizing vasculitides. These results are either recently published or in press. In particular, we continue to prospectively follow the largest group of patients with Wegener's granulomatosis in the world and have established a greater than 90% remission rate with the use of cyclophosphamide. In addition, we have most recently published similar striking results with cyclophosphamide in systemic necrotizing vasculitis of the polyarteritis nodosa group.

In addition, we are prospectively following the largest group of patients in the world with the idiopathic hypereosinophilic syndrome (HES). We have been carrying out intensive investigations concerned with the proper categorization of this heterogeneous group of essentially idiopathic disorders which have as their common denominator serious tissue damage resulting from or associated with their eosinophilia. We have instituted novel therapeutic protocols for the treatment of a subset of these patients with corticosteroids and hydroxyurea. This has resulted in striking remissions in several patients. The relationship between the eosinophilia and the major cause of morbidity and mortality in these patients, i.e. endomyocardial fibrosis is under active study. We have determined that there likely exists in these patients a hypercoagulable state which may explain the high incidence of mural thrombi associated with or even contributing to the myocardopathy. We have demonstrated that a subset of patients have mononuclear "feeder" layers in colony forming unit (CFU) assays which are responsible for their increased eosinophil colony activity and which can impart this abnormally high activity upon eosinophil colony activity of normal individuals. Such studies have major implications in our understanding not only of this particular syndrome, but of the regulation of eosinophil kinetics and the mechanisms of effector function of normal eosinophils.

Proposed Course:

These projects will continue along the lines which have been described above.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00057-07 LCI

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Basic Studies on Pathogenic Fungi

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K.J. Kwon-Chung	Senior Investigator	LCI	NIAID
OTHER:	J.E. Bennett	Head, Clinical Mycology Sec.	"	"
	Itzhack Polacheck	Visiting Fellow	"	"

COOPERATING UNITS (if any)

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V.J. Hearing (NCI)

LAB/BRANCH
Laboratory of Clinical Investigation

SECTION
Clinical Mycology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 5	PROFESSIONAL: 2	OTHER: 3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The interests and proposed course of our unit are to cover basic and applied aspects of various pathogenic fungi including their morphology, taxonomy, pathology, epidemiology, biochemistry and genetics. Topics of present interest include: 1) structure of the capsular polysaccharide of serotype B *Cryptococcus neoformans*, 2) canavanine sensitivity in four serotypes of *C. neoformans*, and 3) melaninless mutants of *C. neoformans*.

Project Description:

Objectives:

Objectives of the project cover both basic and applied aspects of various pathogenic fungi including their morphology, taxonomy, pathogenicity, life cycle, biochemistry and genetics. The topics of present interest include: 1) chemical structure of capsular polysaccharide of serotype B of *Cryptococcus neoformans*, 2) canavanine sensitivity in all four serotypes of *C. neoformans*, and 3) virulence of *mel*$^-$ mutant of *C. neoformans* for mice.

Methods Employed:

Production of Capsular Polysaccharide: Cultures were grown on Sabouraud dextrose broth, and cells were killed by adding buffered formalin and removed by centrifugation. The supernatant was treated with sodium acetate, acetic acid and 95% ethanol to precipitate polysaccharide. The polysaccharide was dissolved in distilled water and treated with sodium acetate and acetic acid. The solution was deproteinated with chloroform and butanol. The aqueous solution was treated with sodium acetate, acetic acid and ethanol. The precipitate was redissolved in distilled water and reprecipitated with 95% ethanol. The polysaccharide was chromatographed on a column of DEAE-cellulose using 0.01 M phosphate buffer, pH 7.3, and a linear salt gradient of 0-1 M NaCl. The 80% of material was eluded at a salt conc. 0.2 M, and this material was dialized and freeze dried.

Chemical Structure of the Polysaccharide: Gel filtration, ultracentrifugation, paper chromatography, hydrolysis by 1 N HCl, methylation, periodate oxidation and chromium trioxide oxidation methods were used to analyze the chemical structure of the polysaccharide.

Tests for Canavanine Sensitivity: Canavanine concentration ranging from 1 μg/ml to 3 mg/ml in yeast nitrogen base agar was prepared, and cells from four serotypes of *C. neoformans* were plated on the medium. The liquid medium was also prepared for the measurement of growth rate.

Isolation of *mel*- Mutant: Cells of *C. neoformans* serotype D were irradiated with ultraviolet light and plated on caffeic acid or L-Dopa agar to isolate the *mel*$^-$ mutant.

Major Findings and Significance to Biomedical Research and the Program of the Institute:

1) Significant Findings in the Area of Polysaccharide Chemistry: The capsular material from the isolate of serotype B has been separated into essentially two fractions. The major fraction which comprises 60% of the total was studied. The results were consistent with a structure having nonreducing D-xylosyl as well as D-glucosyluronic acid groups attached to O-2 of D-mannosyl residues linked α-(1→3) in a linear backbone. Every third D-mannosyl residue is doubly substituted with a D-xylosyl group at O-2 and

a D-glucosyluronic acid group at 0-4.

2) Findings in the Area of Biochemistry: Canavanine sensitivity was found to be related to the serotypes of the cultures. Isolates of serotypes B and C were 100% resistant. Isolates of serotype D were 100% sensitive. The isolates of serotype A, however, were mixed in that 70% were resistant while the remaining 30% were sensitive. This phenomenon together with the creatinine metabolism pattern can be used for the serotyping of *C. neoformans* without the serological tests. Phenoloxidase of *C. neoformans* was found to be a membrane-bound, constitutive enzyme.

3) Findings in the Area of Genetics and Pathogenicity: A mutant with genetic markers ts^- (temperature sensitive) and mel^- (melanin lacking) was found to be completely avirulent for mice or rats. The progeny obtained from the cross between this mutant and the wild type showed no linkage between mating type and either *ts* or *mel* loci, but loose linkage was seen between *ts* and *mel* loci. The virulence of selected progeny showed that *mel* locus plays a major role for the virulence of the isolates.

Proposed Course:

The biochemical aspects of phenoloxidase from *C. neoformans* will be studied. The virulence factor of the fungus will be studied by isolating the natural *mel*$^-$ variants.

Publications:

1. Bhattacharjee, A.K., Kwon-Chung, K.J. and Glaudemans, C.P.J.: On the structure of the capsular polysaccharide from *Cryptococcus neoformans* serotype C II. *Molecular Immunology* 16:531-532, 1979.

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3. Bhattacharjee, A.K., Kwon-Chung, K.J. and Glaudemans, C.P.J.: The structure of the capsular polysaccharide from *Cryptococcus neoformans* serotype D. *Carbohydrate Research* 73:183-192, 1979.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00058-06 LCI

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Pathogenesis and Chemotherapy of Herpesvirus Infections in Man

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S.E. Straus	Senior Investigator, MVS	LCI	NIAID
Other:	R. Berg	Clinical Associate	"	"
	H. Takiff	" "	"	"
	H.S. Aulakh	Fogarty International Fellow	"	"

COOPERATING UNITS (if any)

J. Costa and P. Howley (LP, NCI), G. Vande Woude (LMV, NCI), J. Hay (USUHS), S. Houff, and W. Wallen (LID, NINCDS), and R. Whitley (Birmingham, Alabama).

LAB/BRANCH

Laboratory of Clinical Investigation

SECTION

Medical Virology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 2-1/2	PROFESSIONAL: 1-1/2	OTHER: 1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The pathogenesis, molecular biology, immunology, natural history, and therapy of human herpes virus infections are being investigated. Immunocompetent and immunodeficient patients with a wide range of herpes virus infections have been identified, follow clinically, diagnosed by virus isolation, studied immunologically and treated with systemic antiviral chemotherapy (adenine Arabinoside or acyclovir) when appropriate.

A major effort to study the molecular biology and latency of varicella zoster virus DNA has been initiated. Thermal elution studies, DNA hybridizations, restriction endonuclease analysis, and electron microscopy have been utilized and are shedding light on the structure of the viral DNA.

Project Description:

Objectives:

1) To determine the pathogenesis and natural history of herpes virus infections in both normal and immune compromised humans.

2) To evaluate the efficacy of antiviral agents in the treatment of human herpes virus infection. These infections include both minor and serious diseases produced by herpes simplex types 1 and 2, varicella-zoster virus, cytomegalovirus, and EB virus.

Methods Employed:

1) Patients with suspected herpes virus infections are referred to the Medical Virology Section from the Clinical Center, the National Naval Medical Center, local hospitals and practicing physicians. The patients are evaluated and appropriate specimens are collected for virus isolation and serologic testing.

2) Virus isolation is performed in primary or continuous human or simian cell lines. The viruses are identified by the appearance of typical cytopathic changes and typed by immunofluorescent procedures where required.

3) In selected cases, the viral DNA has been purified, digested by restriction endonucleases and analyzed by agarose gel electrophoresis.

4) The clinical efficacy of adenine arabinoside, adenine arabinoside monophosphate, acyclovir and ribavirin is being investigated in the treatment of herpes virus infections. This unit is collaborating in an NIAID-sponsored multicenter trial evaluating antiviral agents in the treatment of herpes simplex encephalitis, herpes zoster infection of the immune compromised host, chronic mucocutaneous herpes simplex infection, and neonatal herpes simplex infection. Individual studies carried out under the auspices of this section have included evaluations of antiviral drugs in the treatment of selected cases of cytomegalovirus pneumonia, life-threatening Epstein-Barr virus infection, and recurrent genital herpes infection.

Major Findings:

1) During the past year approximately 60 patients have been identified and examined at the NIH with a wide variety of herpes virus infections. Approximately 40 of these patients have had recurrent genital herpes virus infections. Attempts to definitively diagnose the infections by virus isolation have been made in all instances and have been successful most of the time. Over 1,000 virus cultures have been performed with specimens obtained from these patients. In selected individuals numerous specimens have been obtained to quantitatively assess virus shedding during the course of natural and treated infections and provide material for studies of the biology of the viruses and of their latency.

2) Immunological studies: All patients have been studied in a general way to determine whether lymphocyte, immunoglobulin, or complement deficiencies are present. Five patients with severe infections have been studied exhaustively. Two patients were found to have severe combined immunodeficiencies. One of these individuals was diagnosed as having congenital nucleoside phosphorylase deficiency. Two individuals with chronic infectious mononucleosis have been found to have subtle disorders of T cell responsiveness to EB virus in vitro. One individual with EB virus encephalitis failed to amount an appropriate T cell response to his infected B cells. In vitro he demonstrated deficient natural killer cell activity.

3) Clinical trials of anti-viral agents:

a) Herpes simplex encephalitis - a collaborative trial of Ara-A for herpes encephalitis has been completed and data are currently being analyzed. A new protocol to compare the efficacy of Ara-AMP or acyclovir with Ara-A has been approved and will be initiated shortly.

b) Varicella-zoster virus infections: Patients will continue to be treated in the NIH Clinical Center under the collaborative Ara-A study. One leukemic child with primary disseminated varicella infection who failed to respond to Ara-A therapy was treated unsuccessfully with acyclovir. One other adult immunocompromised patient with two separate episodes of disseminated zoster infection was treated unsuccessfully with Ara-A but responded promptly to acyclovir therapy on each occasion.

c) Mucocutaneous herpes simplex infection - immunodeficient patients with chronic mucocutaneous herpes simplex infections have continued to be treated under the collaborative Ara-A study. Two individuals who have failed to improve on Adenine Arabinoside were successfully treated with acyclovir. Patients with genital herpes infection are currently being collected and studied to be incorporated in a forth coming topical trial of ribavirin.

d) Cytomegalovirus infection - One immunodeficient patient with culture proven cytomegalovirus pneumonia who failed therapy with Ara-A and immune serum improved dramatically following acyclovir therapy.

e) Epstein-Barr virus infection - One patient with severe EBV encephalitis improved and has returned to nearly normal neurologic status following therapy with acyclovir.

4) Characterization of varicella zoster virus DNA - Varicella zoster virus has been recovered directly from vesicle fluid specimens of patients with varicella and with zoster infection, and from passaged virus grown in cultures of human embryonic lung cells. Viral DNA purified from these samples has been characterized by several techniques. Thermal elution studies of in vitro labeled DNA have revealed Tm's averaging 88.8°C and permit calculation of a guanine plus cytosine content of 47.6%.

Restriction endonuclease digestion and agarose gel electrophoresis have revealed distinctive cleavage patterns which reflect upon the internal structure of the DNA, permit an estimation of a total genome weight of seventy-seven to eighty million daltons, and provide the basis for future molecular epidemiological investigations. DNA preparations have also been spread and examined by electronmicroscopy. EM measurements have substantiated the above estimated genome weight and have further shed light on the secondary and tertiary structure of the DNA. Additional structural features of varicella zoster DNA would be more easily obtained after genome fragments have been successfully cloned. We have recently obtained approval to clone VZV-DNA in lamdaphage in this laboratory, in collaboration with Dr. George Vande Woude. Finally we have begun a series of DNA reassociation kinetic studies searching for latent varicella zoster virus DNA sequences in human autopsy tissues.

Significance to Biomedical Research and the Program of the Institute:

Herpes viruses produce substantial morbidity and mortality each year. Normal adults and children suffer individual or recurrent infections caused by members of the herpes virus family. These infections range from rather benign afflictions to those such as herpes simplex encephalitis which when untreated causes death in 60-70% of individuals. Immune compromised individuals, of which there are an ever increasing number and variety, are especially susceptible to herpes infections. The above studies have expanded current knowledge of the natural history, pathogenesis, and biology of these infections. The therapeutic trials in which we collaborate have been the first to clearly demonstrate efficacy of antiviral compounds for these types of infections. These results have been encouraging and pave the way for future studies with even more potent antiviral drugs.

Proposed Course:

During the next year we will continue to see patients with debilitating or life-threatening herpes virus infections. We will continue to search for immune abnormalities which underly some of these infections. Antiviral trials will be expanded to examine in controlled studies the efficacy of Ara-AMP, acyclovir and ribavirin in addition to Ara-A.

We will attempt to clone varicella zoster virus DNA fragments, further define the DNA structure, and generate restriction endonuclease maps of the DNA. We will hopefully complete our studies of the latency of varicella zoster virus in humans by performing definitive DNA reassociation kinetic analysis and blot hybridizations of human organs searching for VZV-DNA sequences.

Publications:

1. Cupps, T.R., Straus, S.E., and Waldman, T.A.: Recovery of an immunodeficient patient with recurrent stimultaneous cytomegalovirus pneumonia, disseminated varicella zoster virus and chronic mucocutaneous herpes simplex virus infections following treatment with acyclovir (acycloguanosine). Am. J. Med. In Press.

2. Straus, S.E., Aulakh, H.S., et al: Structural studies of varicella zoster virus DNA. Abstract presented at the Fifth International Herpes Virus Workshop, Cold Spring Harbor, New York, August, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 A1 00154-05 LCI

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Immunologic, Neurophysiologic, Biochemical and Cellular Events in Immediate Hypersensitivity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Michael A. Kaliner	Senior Investigator	LCI,	NIAID
Other:	J. Shelhamer	Clinical Associate,	LCI,	NIAID
	L. Steel	Staff Fellow	"	"
	H. Oertel	Visiting Fellow	"	"
	M. Donlon	Guest Worker	"	"
	S. Wescott	Biol. Lab. Tech.(Biol.)	"	"
	G. Myers	" " " "	"	"
	P. Davis	Clinical Associate	PMB	NIAID
	Z. Marom	Guest Worker	LCI	NIAID

COOPERATING UNITS (if any)

Walter Reed Army Medical Center (R. Evans, R. Summers, L. Smith, and R. Sigler).

LAB/BRANCH
Laboratory of Clinical Investigation

SECTION
Allergic Diseases Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Human asthma and rhinitis involve the excessive secretions of mucus. Cultured human airways incorporate radiolabeled molecules into newly synthesized mucus permitting monitoring of mucous secretion. Human lung mucus has been characterized biochemically and the immunologic as well as neuropharmacologic control of mucous secretion defined. The site and control of human lung parenchyma versus airway prostaglandin production has been identified and the factors generated by anaphylaxis causing prostaglandin synthesis isolated. The histologic responses to rat mast cell granules have been characterized both in rat and monkey skin and the factors responsible for eliciting the inflammation isolated and characterized. The effects of steroids on late phase allergic reactions has been examined. The relationship between calcium influx and rat mast cell degranulation has been analyzed as have the responses of mast cells to histamine stimulation. The autonomic and autocoid responsiveness of subjects with asthma, allergic rhinitis and cystic fibrosis has been studied. An assay for urinary histamine has been developed and the normal range of histamine established.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

The goals of the Allergic Diseases Section are to expand our knowledge of all aspects of immediate hypersensitivity reactions. Our particular expertise and focus are defining the biochemical events which accompany and control the triggering of allergic reactions, the neurophysiologic mechanisms which potentially modulate these events, the characterization of the mediators of anaphylaxis and identification of the responses of relevant target issues to these mediators.

We are concentrating on three primary areas: 1) employing human lung tissue, we study "asthma in a test tube" in order to understand the immunologic triggers, biochemical concomitants and neurophysiologic controls of mediator release; the identification of additional mediators of anaphylaxis and the study of mucous secretion. 2) Rat peritoneal mast cells are utilized as a pure source of effector cells which may be readily studied in vitro and may amplify as well as initiate new areas of interest in relationship to the human lung model and 3) Clinical allergic asthma is being evaluated in an extensive protocol designed to expand our understanding of the neurophysiologic mileau of allergic individuals as compared with cystic fibrosis and normal subjects.

Methods Employed:

1. Human lung model: Lungs removed, usually for cancer resection, almost always contain large amounts of normal, functioning lung tissue. We obtain these lungs in cooperation with the surgical and pathology departments of all the major hospitals in the Bethesda area. The lung tissue is washed, fragmented and replicated into 200 mg samples and subsequently incubated in dilutions of allergic serum for 2 hours at 37°C for 18 hours or for 2 hours at 25°C. After this "sensitization" period, the serum is removed and the fragments challenged with allergen. The interaction of the allergen with tissue (mast cell) bound IgE induces the release of histamine, slow-reacting substance of anaphylaxis (SRS-A), eosinophil chemotactic factor of anaphylaxis (ECF-A), and prostaglandin E_1 and $F_{2\alpha}$ (PGE_1 and $PGF_{2\alpha}$). These mediators may be assayed on the isolated terminal portion of the guinea pig's ileum (histamine, SRS-A), chemotactic chambers (ECF-A) or by radioimmunoassay (PGE_1, $PGF_{2\alpha}$).

We have been determining alterations in the tissue levels of cyclic AMP and cyclic GMP after treating lung with various agonists in order to define the role these nucleotides play in modulating the release of mediators. Cyclic AMP may be measured by either a protein kinase binding assay or radioimmunoassay while cyclic GMP is determined by radiommunoassay after acetylation or succinylation.

Mucous glycoproteins are monitored by incorporating ^{3}H-glucosamine into the mucous glands during an 18 hour incubation period. Secretions are dialyzed against 6M urea, filtered over sepharose 2B, and analyzed by ion exchange chromatography. Mucous glycoproteins may also be hydrolyzed by $NaBH_3$ or be focused on a sucrose gradient in the presence of ampholytes.

2. Rat mast cells: rat peritoneal and pleural mast cells are obtained from freshly sacrificed male Sprague-Dawley rats by lavage of these cavities. The peritoneal cells include 5% mast cells (or approximately 500,000 to 1 million/animal). The cells are used either in mixed cell suspensions or after purification by centrifugation into albumin cushions. Mast cell granules are isolated with intact perigranular membranes by sonication and centrifugation through sucrose cushions. Granules free of membranes are obtained by osmotic lysis of mast cells.

Calcium studies with rat mast cells involve incubation of cells with ^{45}Ca, followed by rapid centrifugation through silicone oil into water. Histamine released into the upper layer is assayed by an automated fluorometric assay while ^{45}Ca associated with the cells spun into the bottom layer is quantitated by scintillation.

Receptor studies involving mast cells employ standard methodology - incubations with radiolabeled ligands at 4^{o}C in the presence of an antagonist followed by rapid filtration.

3. Clinical asthma study: The methods employed in this study are covered in detail in the clinical protocol and include: a) measurement of cutaneous blood flow by ^{133}xenon disappearance; b) pupillary responses as measured by a binocular pupillometer; c) airway obstruction as measured by flow-volume curves with and without helium inhalation; d) serum cyclic nucleotide responses as measured after intravenous isoproterenol administration.

An additional study completed this year involved examining the effects of histamine infused into normal subjects in comparison to mild allergic asthmatics. Techniques employed consisted of monitoring pulse rate, blood pressure, peak expiratory flow, skin test responses, skin temperature, urinary histamine, plasma histamine, plasma cyclic nucleotides and plasma prostaglandins.

Major Findings:

1) Human Lung Studies

A) Human airways cultured in CMRL 1066 may be maintained in organ culture in an atmosphere of 50% O_2, 45% N_2 and 5% CO_2 for at least two weeks. These fragments incorporate ^{3}H-glucosamine into mucous glycoproteins which may be analyzed by ethanol precipitation and membrane filtration. A complete analysis of the effects of arachidonic acid upon mucous release has been completed. Many prostaglandins, including $PGF_{2\alpha}$, $PGF_{1\alpha}$, PGA, PGB, and PGI_2 (1-100 µM) increase mucous secretion with the maximum effect seen at 1 µM. PGE_1 also increases mucous release while PGE_2 inhibits release (maximal effect at 1 µM). Arachidonic acid (1-100 µM) enhances mucous release suggesting that derivatives such as prostaglandins might be responsible. However, both aspirin (100 µg/ml) and indomethacin (10 µg/ml) enhance mucous release while preventing prostaglandin formation. Therefore, either the inhibition of PGE_2 suppression of mucous release is the primary action of NSAID or lipoxygenase products are responsible. ETYA (1-100 µM) inhibited mucous release and PG formation. NDGA, Vitamin A and α Naphthol each enhanced mucous release suggesting that lipoxygenase products are the enhancing agents. A mixture of monohydroxy arachidonic acids were synthesized, including 8, 11, 12, and 15 HETE. This mixture of HETE's enhanced mucous release in a dose-related manner at concentrations of 100-200 nM. Finally, HETE added to ETYA resulted in marked synergism. Therefore, a lipoxygenase product of arachidonic acid, probably HETE, augments mucous release.

B) Prostaglandin formation by human lung after anaphylaxis may in part be attributed to histamine. However, about 50% of the prostaglandin formation is independent of histamine. Several factors released from human lung during anaphylaxis are able to cause guinea pig lung parenchymal preparations to produce thromboxane B_2, $PGF_{2\alpha}$ and PGE. We have characterized these factors and now recognize that they consist of two major components. The larger is retained on a DM5 filter (> 5000 MW), and separates on a Sephadex G-150 column into two factors with apparant molecular weights of >200,000 and 55,000 daltons. The second component filters through DM5 but is retained by UM05 filters (MW = <5000 but >5000), filters on Sephadex G-25 with an apparent molecular weight of 1500, adheres to DE52 in 0.001 of NH_4HCO_3, pH 7.8, and elutes in one major peak at 0.1M NH_4 HCO_3. Thus, human lung undergoing anaphylaxis generates at least three molecules in addition to histamine which are capable of causing prostaglandin synthesis. The low molecular weight fraction has been termed prostaglandin generating factor of anaphylaxis (PGF-A) and has been subjected to paper and thin layer chromatography, cation exchange chromatography and amino acid analysis. This molecule consists of 13 amino acids and is resistant to trypsin. PGF-A requires the presence of calcium but not magnesium to act, is maximally effective at 37°C and produces peak PG generation within 5 minutes at pH 7.4. The exact mechanism of action is under investigation.

2) Rat Mast Cells

Mast cell granules injected into the skin of rats and monkeys were found to cause significant inflammatory reactions. Granules isolated with intact perigranular membranes caused a polymorphonuclear-rich infiltrate apparent in 2 hours which peaked by 8 hours. Subsequently, an intense mononuclear infiltrate replaced the PMN's and peaked at 24-48 hours. Granules isolated free of membranes and washed free of elutable mediators injected into skin caused a somewhat less pronounced polymorphonuclear infiltrate and a somewhat greater mononuclear infiltrate. The responsihle factors were found in close association with the granule matrix. In fact, granule preparations washed free of elutable meditors were found to have three separable factors capable of inducing these infiltrates. These factors may be separated on filters - the two larger factors are retained on a UM10 filter. The larger molecules have apparent molecular weights of 200,000 and 70,000 daltons respectively on gel filtration through Sephadex G-200. The smaller molecule chromatographs on Sephadex G-25 with a apparent MW of 1250. The smaller molecule binds to both anion and cation exchange columns and eluates as one major peak in a salt gradient. The larger molecules may be precipitated by 10% ethyl alchohol but not by $(NH_4)_2SO_4$. Therefore, there are three factors constituent within the mast cell granule which elicit delayed inflammatory responses in rat skin. The factors have been termed Inflammatory Factors of Anaphylaxis, IF-A. The low molecular weight IF-A has been examined for its amino acid content and contains 15 amino acids.

The effects of corticosteroids on the infiltration induced by IF-A has been examined. Adrenalectomy had no effect while hydrocortisone or dexamehtasone (at twice their maintenance concentration) prevented the inflammation. Methylprednisone was also effective but at much higher concentrations. Hydrocortisone inhibited the inflammation within 8-24 hr. Therefore, the inflammatory response elicited by IF-A is steroid responsive.

b) Initiation of rat mast cell secretion involves transmembrane movement of extracellular calcium. The equilibration with extracellular calcium in resting mast cells is extremely rapid, being completed within 60 sec at 37°C in the presence of 0.8 mM calcium. Analysis of calcium movement into the rat mast cell undergoing a secretory event reveals that uptake is increased within 5-10 sec, only 50% is removed by subsequent exposure to EGTA and the time course of calcium uptake continues after granule release is completed. Calcium uptake was initiated by phosphatidyl serine (PS) added to the bathing medium in the absence of histamine release. As PS augments antigen-induced histamine release, the calcium translocation observed may relate to this property. ATP (10 mM) inhibits mast cell degranulation and chelates calcium; at 1 mM, ATP induces release and stimulates calcium uptake. ATP does not require hydrolysis to ADP or AMP for this response and there is insufficient ATP released by the mast cell to suggest that secreted ATP has any influence on the release reaction. Further characterization of the role which calcium plays in degranulation by mast cells is ongoing.

Amiloride is a diuretic whose mechanism of action involves competitive inhibition of a sodium-calcium antiport exchange system. Amiloride induces a dose-dependent (100-1500 mM) release of histamine from rat mast cells. This action is markedly potentiated by depletion of sodium from the bathing solution indicating competition for the sodium antiport. Amiloride acts independently of extracellular calcium, probably by displacing intracellular calcium.

c) Rat mast cells contain 0.3-0.5 p moles cyclic AMP/10^6 cells and > 0.010 p moles cyclic GMP. Histamine (up to 10^{-2}M) fails to increase cyclic AMP levels unless aminophyllin is present. Aminophylline at 5 x 10^{-5} or 5 x 10^{-4} gives a dose-related increase in histamine (5 x 10^{-3}m) increases in cyclic AMP. Histamine acts within 15 sec to increase cyclic AMP. Employing ^{3}H-serotonin labeled rat mast cells, histamine alone was found to have no effect on anti-IgE induced degranulation. However, histamine in the presence of aminophylline inhibited serotonin release. Neither dimaprit nor impromidine, two selective H-2 agonists, increased cyclic AMP or inhibited mast cell degranulation. However, 2-methyl histamine and 2-pyridylethylamine, two specific H-1 agonists, both increased cyclic AMP and inhibited serotonin release. Therefore, rat mast cells respond to H-1 stimulation with increases in cyclic AMP and inhibition of degranulation. Preliminary evidence indicates the presence of H-1 receptors but not H-2 receptors on mast cells.

d) Mast cells have been examined for the presence of beta adrenergic receptors; 85000/cell high affinity (K_D=7.2 nM) beta adrenergic receptors have been located. In addition, 320000/cell, low affinity receptor have also been found. A significant increase in the number of receptors after degranulation was observed. As the granule membrane is added to the cell membrane during exocytosis, the presence of beta adrenergic receptors on the perigranular membrane was sought. Each granule contains 34 high affinity receptors (7.2 nM K_D) indicating that the increase in receptor number may reflect the addition of perigranular receptors to the cell membrane. No alpha adrenergic or muscarinic receptors were detected.

3) Clinical Studies

a) The autonomic responses of asthmatic subjects have been compared with those of subjects with allergic rhinitis and normal controls. Alpha adrenergic function was assessed by pupillometry and cutaneous vascular responses. In both instances, the subjects with asthma were significantly more sensitive than both allergic rhinitis and normal control subjects. Beta adrenergic function has been analzyed by the cardiovascular and cyclic AMP responses to intravenous isoproterenol in these subjects. Patients with either asthma or allergic rhinitis have significant impairment of both beta adrenergic responses as compared with controls. Parasympathetic function has been analyzed by pupillometry, sweat responses and bronchial challenge. Again, patients with either allergic rhinitis or asthma demonstrate the same degree of exaggerated responsiveness in comparison with normal controls. Therefore, asthmatics are selectively more responsive to alpha adrenergic stimulation while both asthmatics and subjects with allergic rhinitis have increased cholinergic and diminished beta adrenergic responses.

Subjects manifesting reproducibly positive allergic skin tests but who are asymptomatic have also been examined. These subjects who are "pre-allergic" have reduced beta adrenergic, hyper-responsive cholinergic and normal alpha adrenergic responses much as the subjects with allergic rhinitis. Taken together these data indicate that reduced beta adrenergic responsivity is associated with the atopic state as is excessive cholinergic responsiveness. However, excessive alpha adrenergic responses are seen only in asthmatics.

More recently, we have completed an examination of 11 young adult subjects with cystic fibrosis and 9 parents (obligate heterozygotes) of cystic fibrosis children. The CF subjects were significantly more sensitive than asthmatics in regards to both alpha adrenergic and cholinergic responsiveness and equally abnormal in regards to depressed beta adrenergic responses. Only 3 of the CF's were concomitanly allergic and these 3 were relatively less abnormal than the other 8. Therefore, the autonomic abnormalities uncovered may be found in diseases other than allergy and may relate to glandular (mucus) secretion.

The obligate heterozygotes were, as a group, abnormal in both alpha adrenergic and cholinergic responses, although much less so than the affected children. This observation suggests that the hyper-responsiveness is inherited as a primary defect rather than appearing as a secondary phenomena.

The presence of antibodies against beta adrenergic receptors in the plasma of asthmatics was measured employing canine lung membranes. Antibodies were detected using immune precipitation of solubulized membranes and specific depletion of binding with immunosorbant techniques. Four of 19 asthmatics, 3 of 25 normal subjects, 1 of 13 allergic rhinitics and 1 of 9 cystic fibrosis patients had antibodies. Of the subjects with antibodies most were markedly abnormal in regards to beta adrenergic responsiveness; thus, 3/4 subjects who were the least responsive to isoproterenol had antibodies. The 3 normal control subjects who had antibodies to their beta adrenergic receptors had abnormal beta adrenergic responsiveness as well. Therefore, antibodies to beta adrenergic receptors may influence autonomic responsiveness.

b) The wheal and flare skin test was examined in order to decide whether both histamine H-1 and H-2 receptors contribute to the increased vascular permeability. As judged by the effect of hydroxyzine and/or cimetidine pretreatment, only H-1 effects were appreciated. Histamine infused from 0.05-1 μg/kg/min for 30 min in sequentially increasing doses had no effect on skin test responses to histamine, compound 48/80 or antigen.

The physiologic responses to histamine were also assessed. Suprisingly, the asthmatic subjects required significantly more histamine for the induction of cutaneous flushing, headaches, increases in pulse rate and pulse pressure. Thus asthmatics are abnormally insensitive to histamine.

c) An assay for urinary histamine was developed. The determination involves a comparison between urine with and without diamine oxidase treatment,followed by cation exchange chromatography, n-butanol extraction, O-phthalaldehyde condensation and fluorometric assay. Males and females excrete equivalent amounts of histamine, spot urines provide results as useful as 24 hr collections and histamine in frozen urine is stable. Normal urine histamine is 10 ng/ml or 1-2 μg/day. The availability of measuring urinary histamine has proven useful in diagnosing episodes of anaphylaxis and may be essential to the differential diagnosis of cutaneous flushing disorders. Several collaborative studies are in progress.

Significance to Biochemical Research and the Program of the Institute

Increased understanding of the mechanisms of immediate hypersensitivity and its control may eventually allow improved therapy of the 44 million Americans with allergic rhinitis, the 9 million with asthma and the 22 million with urticaria. The model system employed permit sophisticated analysis of many of the aspects involved *in vivo*. Unquestionably, our analysis of mucous secretion will enable us to approach clinical problems of bronchorrhea (asthma, cystic fibrosis, chronic bronchitis) with a greater understanding as well as permit *in vitro* analysis of new modalities of therapy.

The observation that mast cell granules may elicit delayed inflammatory responses may provide the mechanisms for explaining certain long-observed but hitherto poorly-understood clinical problems such as: the epithelial destruction accompanying asthma, the chronic inflammation associated with perennial rhinitis, the hyperreactive airways disease occurring in asthma and delayed responses seen with allergy skin tests. Isolation of a low molecular weight molecule eliciting this late phase response implies potent chemotactic properties and should allow studies of the cellular mechanisms of chemotaxis employing a biologically-relevant molecule.

Characterization of prostaglandin generating factors elaborated by human tissues undergoing biologic processes (allergic responses) will permit identification of the role these factors play in a variety of diseases including asthma. Further, purification of these factors will permit a detailed analysis of the mechanisms of prostaglandin synthesis.

Analysis of the autonomic responsiveness of allergic and cystic fibrosis patients enables us to approach these diseases with a unique appreciation of the capacity of neurophysiologic processes to modulate and complicate these diseases. Clearly the abnormalities uncovered have therapeutic implications in both the CF and asthma populations. The detection of antibodies to the beta adrenergic receptor has far-reaching consequences for our understanding of abnormalities of autonomic function.

The abnormal autocoid responsiveness in asthma was unexpected and its meaning is still not clear. However, the presence of abnormal autocoid and autonomic responses in asthma indicates that this disease is even more complex than previously appreciated.

Finally, the availability of a sensitive assay for urinary histamine should markedly facilitate our examination of the role histamine plays in human health and disease.

In summary, the Allergic Diseases Section is approaching immediate hypersensitivity at several levels with interests in both clinical and basic areas. This approach is providing a mileau in which results are rapidly transmitted from the laboratory bench to the clinic and from which a finer appreciation of disease processes is evolving. The direction in which the lab is headed should continue to translate basic observations to enhanced clinical practice.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 A1 00155-05 LCI

PERIOD COVERED October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Phagocytic Cell Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Laboratory of Clinical Investigation

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Bacterial Disease Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4 1/2	4 1/2	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The mechanism of leukocyte activation by chemotactic factors has been studied using electrophysiology, fluorescent probe, surface charge and ultrastructural techniques in leukocytes from normal subjects and patients with abnormal phagocytes. A severe defect in elicited membrane potential charges in neutrophils from patients with chronic granulomatous disease has been seen.

Studies assessing the mechanism of modulating leukocyte locomotion indicate that limited secretion of specific granules, which accompanies chemotaxis, is associated with increased cell adhesiveness and increased availability of chemoattractant receptors. Limited secretion may be required for neutrophil margination in vivo.

Studies of the two populations of neutrophils we had identified previously indicate that during the neutropenia that follows in vivo endotoxin or hemodialysis, a subpopulation of neutrophils with poorly demonstrable Fc receptors is the predominant neutrophil left in the circulation. Clinical studies assessing the effect of pharmacologic agents on the neutrophil subpopulations in normal subjects, patients with recurrent infection and host defense defects are underway.

Project Description

Objectives:

1) Study the mechanism of leukocyte chemotaxis.

2) Study the phenomenon of deactivation of leukocyte chemotaxis and to relate this to the pathophysiology of leukocyte dysfunction syndromes.

3) Develop new techniques to quantitate chemotaxis and the spreading of living phagocytes.

4) Study the role of cations in leukocyte activation with particular emphasis on the utilization of indirect probes of membrane potential to study the relationship between changes in membrane potential and initiation of neutrophil motility, phagocytosis, degranulation and superoxide generation.

5) Study neutrophil subpopulations in normal subjects and in patients with neutrophil dysfunction.

6) Study the mechanism of neutrophil margination and aggregation at capillary beds prior to cell migration into tissues.

7) Study the mechanism of mobilization and secretion of neutrophil lysosomal granules, study the relationship of the secretory process to neutrophil function and dysfunction and to study how neutrophil secretory products may influence homeostasis.

8) Characterize and define the mechanism of abnormal neutrophil chemotaxis following thermal injury.

9) Study the effects of pharmacologic agents on leukocyte function *in vitro* and *in vivo*.

10) Assess the effect of histamine on neutrophil function.

Methods Employed

Peripheral blood leukocytes were separated from heparinized whole blood by dextran sedimentation and Hypaque-Ficoll separation. Chemotaxis was evaluated using a radioassay employing ^{51}Cr labeled leukocytes and a double micropore filter system or a single filter measuring the distance migrated into the filter by the population of responding cells. Cell adherence was measured by quantitating the number of leukocytes adhering to plastic surfaces or to nylon wool columns. Phagocytosis, bactericidal capacity, nitroblue tetrazolium dye reduction, hexose monophosphate shunt activity, superoxide generation and hydrogen peroxide formation were measured using standard techniques. PMN receptors for the chemoattractant f-met-leu-phe were quantitated as published previously.

The fluorescent probe dipentyloxacarbocyanine and the radioisotope trimethylphosphonium were used as indirect probes of membrane potential changes in neutrophils. Leukocyte surface charge was measured with a Zeiss cytopherometer. Intracellular calcium was localized in human neutrophils by the intracellular precipitation of pyroantimonate anion followed by microprobe analysis and studies with the metal chelators (EDTA and EGTA).

The cytoskeleton of polymorphonuclear leukocytes was studied during conditions of chemotaxis and chemokinesis using 0.45 μm micropore filters. These small filters impede leukocyte migration but permit pseudopod penetration and a fixed orientation of leukocytes is thereby established. Orientation of living cells was monitored by well established techniques. Tyrosylation of the α-chain of tubulin was studied as described previously. Secretion of leukocyte granule enzymes was monitored by standard spectrophotometric assays. Polymorphonuclear leukocyte granules were separated and fractionated using sucrose gradient techniques and various enzyme markers were utilized to identify granule types.

Neutrophils were fractionated into subpopulations based on their ability to rosette IgG coated erythrocytes. In some studies, in normal human volunteers, the effects of intravenous endotoxin on neutrophil subpopulations was examined.

Major Findings

1. Technologic advances have been made for quantitating leukocyte chemotaxis, and bactericidal activity. Using an Optomax image analyzer the amount of time required to quantitate the number of cells migrating a distance into a micropore filter or the number of bacteria on a pour plate has been reduced by 90%. In addition, the image analyzer has made possible a quantitative assay of leukocyte spreading. Preliminary studies indicate the analyzer will make possible the tracking of moving cells thereby providing quantitative information on initial rates of locomotion (Alling, Gallin).

2. During chemotaxis in vitro and exudation in vivo neutrophils secrete their granule contents and this secretion is preferential for specific granules. It has been shown that this secretory event modulates chemotactic responsiveness. With limited exocytosis there is increased chemoattractant binding to the cell membrane and this increased binding of chemoattractants increases the availability of chemoattractant receptors without altered affinity of binding. This increase in the availability of chemoattractant receptors is associated with increases in membrane receptors and increased internalization of receptors. The data indicate the increased receptor availability may be related to translocation of specific granule membrane, which contains the chemoattractant receptors, to the cytoplasmic membrane. This may provide the mechanism for membrane turnover during sustained locomotion and cell margination. Preliminary studies in normal volunteers indicates mobilization of chemoattractant receptors precedes and may be associated with margination. Following vigorous exocytosis both chemotaxis and cell orientation in a gradient of

chemoattractant are inhibited. This inhibition is related to decreased binding of a chemotactic factor to the cell. The latter has been associated with decreased peptide affinity and increased hydrolysis of chemoattractant by granule products secreted extracellularly. (Fletcher, Gallin, Schiffmann).

3. Exocytosis of neutrophil granules in vitro increases neutrophil adhesiveness and aggregation. This has been related to neutralization of the net negative surface charge of the cell membrane and facilitation of cell-cell and cell-surface adhesion. Treatment of neutrophils with neuraminidase or poly-l-lysine also increases their adhesiveness without causing degranulation. In addition, submembranous deposition of cations (calcium) at the leading edge of cells in a gradient of chemoattractant was observed and speculated to facilitate fusion of intracellular granules with the cytoplasmic membrane. In this regard, it is of interest that the specific granules, which are most accessible to extracellular release, are more negatively charged than the azurophil granules. Furthermore lactoferrin, which is found in neutrophil specific granules, facilitates neutrophil spreading and adhesiveness. (Gallin, Cotton, and Cramer)

4. A patient with abnormal neutrophil adhesiveness and spreading, and a missing plasma membrane glycoprotein, has been described (Babior, Gallin).

5. Vitamin K derivatives (vitamin K_3 and vitamin K_5) are effective inhibitors of neutrophil spreading, locomotion, phagocytosis and killing without affecting receptor availability, hexosemonophosphate shunt activity or lactate production. Vitamin K appears to exert its effect by vitamin K epoxide oxidation of vital sulfhydryl groups and possibly vitamin K carboxylation of γ carboxy glutamic acid, a calcium sequestration system. The vitamin K effects on the neutrophil also include inhibition of elicited membrane potential changes, stimulation of calcium release, accumulation of submembraneous calcium and inhibition of the tyrosylation of the α-chain of tubulin. The possibility vitamin K derivatives possessing select antiinflammatory activities exist is being explored (Gallin, Seligmann, Cramer, Nath, Schiffmann).

6. Using fluorescent cyanine dyes and tritiated trimethyl phosphonium ions chemoattractants and degranulating stimuli were shown to stimulate membrane potential changes in neutrophils. The potential changes in neutrophils are similar to those observed in macrophages using direct intracellular recording techniques. The data using degranulating stimuli indicate low concentrations of degranulating stimuli increase f-met-leu-phe receptor efficacy for eliciting membrane potential changes as monitored with a fluorescent dye. Vigorous secretion inhibits receptor efficacy. These latter observations support and extend the data on the effect of degranulating stimuli on chemoattractant receptor availability and on chemotactic responsiveness. In addition the data suggest degranulating stimuli modulate neutrophil locomotory responsiveness by alteration of neutrophil plasma membrane ion permeability, or the concentration of

intracellular ions. Studies with the fluorescent cell sorter have suggested subpopulations of neutrophils exist as assessed with membrane potential sensitive fluorescent probes (Seligmann, Chusid, Gallin).

7. Assessment of chemoattractant elicited membrane potential changes using indirect probes indicate that neutrophils from patients with chronic granulomatous disease (CGD) have a major abnormality. Neutrophils from other patients with phagocyte defects do not show any abnormality of elicited membrane potential changes. Studies of calcium, potassium, sodium and chloride fluxes are currently underway to further characterize the basis of the defect in CGD neutrophils. Likewise intracellular recordings with microelectrodes in CGD monocytes are underway. (Seligmann, Gallin (E.), and Gallin, J.I.)

8. The acquired defect of neutrophil locomotion seen following thermal injury precedes sepsis and has been shown to be a function of burn wound area. The phagocyte chemotaxis defect is linearly related to the degree of lysosome lost from the neutrophil. A rabbit model for thermal injury has been developed to enable further study of this acquired neutrophil defect (Davis, Gallin).

9. Abnormal PMN function in cells from patients with the Chediak-Higashi syndrome is associated with a 300 fold increase in the tyrosylation of the α-chain of tubulin. This represents the first biochemical evidence for abnormal microtubule metabolism in a patient with phagocyte dysfunction (Nath, Gallin).

10. Normal human neutrophils were separated into two populations based on their ability to rosette human IgG coated erythrocytes and tested for their ability to rosette complement-coated erythrocytes. The rosette forming neutrophils had superior function of chemotaxis, and phagocytosis of opsonized particles. Both neutrophil populations formed rosettes with complement-coated erythrocytes equally well. Moreover, neither population of cells displayed those complement receptors felt to be markers of immature granulocytes. Studies of the kinetics of clearance of the two populations of neutrophils from the blood stream are underway in rabbits. (Frank, Cotton, Gallin).

11. Twenty minutes following intravenous administration of E. coli endotoxin to normal volunteers, during the period of neutropenia, the predominant circulating neutrophil is a "subpopulation" without readily demonstrable IgG (Fc) receptors. In related studies, twenty minutes after initiating hemodialysis in patients with chronic Schizophrenia, also during a period of neutropenia, the remaining circulating neutrophils are significantly enriched with a subpopulation of neutrophils with poorly demonstable Fc receptors. The potential clinical meaning of a subpopulation of neutrophils in patients rendered neutropenic by cell margination is being studied (Cotton, Gallin).

12. A group of patients with extreme elevation of IgE and recurrent infections with Staphylococcus aureus and Candida albicans was studied. The IgE in these patients bound to S. aureus and C. albicans. There

appears to be cross reactivity of the specific IgE with each organism. The IgE did not bind to the other organisms to which the patients did not have clinical problems. In related studies, designed to determine if histamine has any effect on neutrophil function, it was found that histamine blocks elicited membrane potential respones and superoxide generation in neutrophils stimulated with the chemoattractant f-met-leu-phe but not by cells stimulated with the calcium ionophore A23187. (Berger, Seligmann, and Gallin).

Significance to Biomedical Research and the Program of the Institute

The accumulation of leukocytes at inflammatory, immune and allergic sites is critical for appropriate responses. Understanding the physiologic basis for events regulating these processes will provide the basis for therapeutic manipulation.

One of the first steps in leukocyte mobilization from the blood stream is increased cell adhesiveness to the endothelium. This is followed by local leukocyte aggregation and then diapedesis. Our finding that limited degranulation and exocytosis of intracellular granules markedly enhances these events, together with the observation that human pyrogen initiates these processes, provides a clue as to what controls margination and then migration of leukocytes from the blood stream. The related observation that vigorous degranulation in vitro inhibits chemotaxis suggested that some acquired chemotactic defects relate to excessive degranulation in vivo. In support of this is our finding that following thermal injury the severity of the acquired chemotactic defect is linearly related to the amount of intracellular lysozyme released. Clinically, it is of interest that the degranulation and defective chemotaxis precedes the severe and often lethal pyrogenic infections that follow thermal injury.

In related studies it appears that lactoferrin, contained in neutrophil specific granules and secreted when cells are activated, facilitates neutrophil spreading and adhesiveness. Thus the neutrophil secreted product lactoferrin may be critical for margination.

Two observations from our studies of the mechanism of the phenomena of degranulation, cell adhesiveness and chemotaxis may be particularly important to the cell biology of chemotaxis and perhaps relevant to clinical studies as well. We have shown that leukocyte adherence, aggregation and secretion may be under the modulating influence of electrostatic forces. Development of techniques for controlling these forces in vivo may have potential clinical use. Our data that neutrophil specific granules are a potential source of new cytoplasmic membrane and chemoattractant receptors is intriguing in terms of understanding the basis for membrane turnover during chemotaxis. These studies need to be extended and explored further.

The use of fluorescent carbocyanine dyes to measure membrane potential is emerging as a rapid test of cell reponsiveness that appears to be as reliable and easier to use than other indirect probes of membrane potential. The adaption of this method of the fluorescent cell sorter provides an opportunity to look at individual cells and to better characterize and detect subpopulations of leukocytes. The demonstration of a severe abnormality of chronic granulomatous disease neutrophils using this test may provide a new simple rapid diagnostic test for this disease. In addition, the implications that the observed abnormality in chronic granulomatous disease reflects abnormal ion flux studies, perhaps related to abnormal activation mechanisms, provides new insights into the nature of the defect in chronic granulomatous disease.

The continuation of our studies of neutrophil subpopulations has indicated that one population of cell (PMNs with readily demonstrable Fc receptors) is particularly available for margination. We are presently extending these studies of neutrophil subpopulations to explore their kinetics of circulation, and their functional role. In addition, the effect of various disease states and pharmcologic agents on these two neutrophil populations in man and in animals is under study.

The demonstration that vitamin K derivatives have pronounced effects on neutrophil function may have important implications in terms of understanding normal neutrophil function and also may provide new approaches for pharmacologic manipulation of phagocyte function in inflammatory diseases.

Prospsed Course

We plan to continue some of the studies outlined above.

Publications

1. Cramer, E. B. and Gallin, J. I.: Localization of submembranous cations to the leading end of human neutrophils during chemotaxis. J. Cell Biol. 82:369-379, 1979.
2. Gallin, J. I., Gallin, E. K., and Schiffmann, E. The Mechanism of Leukocyte Chemotaxis. Adv. in Inflamm. Res. 1:123-138, 1979.
3. Klempner, M. S., Dinarello, C. A., Henderson, W. R. and Gallin, J. I. Stimulation of neutrophil oxygen-dependent metabolism by human leuko-cytic pyrogen. J. Clin. Invest. 64:996-1002, 1979.
4. Schiffmann, E. and Gallin, J. I. Biochemistry of Phagocyte Chemotaxis. In B. L. Horecker and E. R. Stadtman (Eds.) In: Current Topics in Cellular Regulation 15:203-261, 1979. New York, Academic Press.
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6. Seligmann, B. and Gallin, J. I. Secretagogue modulation of the response of human neutrophils to chemoattractants: studies with a membrane potential sensitive cyanine dye. Molecular Immunology. 17:191-200, 1980.
7. Davis, J. M., Dineen, P. and Gallin, J. I. Neutrophil degranulation and abnormal chemotaxis following thermal injury. J. Immunol. 124:1467-1471, 1980.

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10. Seligmann, B. E., Gallin, E. K., Martin, D. L., Shain, W. and Gallin, J. I. Interaction of chemotactic factors with human polymorphonuclear leukocytes: studies using a membrane potential sensitive cyanine dye. J. Memb. Biol. 52:257-272, 1980.

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12. Whited, S. C. and Gallin, J. I. Neutrophil chemotaxis. Interna. J. Dermatol. 19:130-138, 1980.

13. Davis, J. M. and Gallin, J. I. The Neutrophil. The Cell Biology of Immunity and Inflammation. Ed. by Oppenheim, J. J., Rosenstreich, D. L. and Potter, M. Elsevier North-Holland. In Press.

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15. Klempner, M. S., Gallin, J. I., Balow, J. E., and Van Kammen, D. P. The effect of hemodialysis and C5a des arg on neutrophil subpopulations. Blood. 55:777-783, 1980.

16. Crowley, C. A., Curnutte, J. I., Rosin, R. E., Andre-Schwartz, J., Gallin, J. I., Klempner, M., Snyderman, R., Southwick, F. S., Stossel, T. P., Babior, B. M. An inherited abnormality of neutrophil adhesion: its genetic transmission and its association with a missing glycoprotein. New Engl. J. Med. 302:1163-1168, 1980.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00188-02 LCI

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Rapid Diagnosis of Infections by Enzyme-Linked Immunoabsorbent Assays

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Stephen E. Straus	Senior Investigator Medical Virology Section	LCI NIAID
Other:	Richard A. Berg	Clinical Associate	" "

COOPERATING UNITS (if any)

J.E. Bennett (LCI, NIAID), R. Brown (LCI, NIAID), P. Pizzo (LPO, NCI), B. Murphy (LID, NIAID), S. Rennard (LDBA, NIDR), P. Charache, and R. Yolken (Johns Hopkins University)

LAB/BRANCH

Laboratory of Clinical Investigation

SECTION

Medical Virology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1	PROFESSIONAL: 1	OTHER:

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Enzyme-linked immunoabsorbent assays have been developed to detect and quantitate a number of viral bacterial and fungal antigens and antibodies as well as DNA. During the past year we have completed studies indicating that influenza A hemagglutinin can be reliably detected in clinical specimens and used as a rapid means of diagnosis. In addition ELISA assays have been successfully used for typing of pneumococci and for evaluating the clinical immune response of splenectomized patients to pneumococcal vaccine.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

1) To develop a rapid assay for influenza infection in order to identify individuals and patient groups which may benefit from amantadine chemoprophylaxis.

2) To develop rapid, more sensitive assays to detect adenoviruses in stool specimens.

3) To develop a convenient assay for the development of antibodies directed against specific types of pneumococci.

4) To develop a rapid assay which would detect and quantitate DNA at the nanogram level.

Methods Employed:

Either direct or competitive assays are employed. Briefly, the desired antigen is absorbed to the surface of plastic microtiter wells. Specific antisera is added and binds to the antigen where present. Development of the antigen-antibody reaction is detected by addition of a second enzyme-linked antibody directed at the first antibody. The reaction is detected colorimetrically by addition of a chromogenic substrate specific for the enzyme.

Major Findings:

1) An ELISA capable of detecting less than 1 hemagglutinin unit of influenza A virus has been developed. The assay has been applied and proven to be sensitive and specific in detecting virus in nasal washings taken following experimental infections in ferrets. More recently we have successfully completed and reported the application of this assay for the rapid detection of influenza A infections in humans[1].

2) Adenoviruses: An ELISA has been developed to reliably detect human adenoviruses. The test utilizes antisera prepared in this laboratory and is currently being applied to the rapid detection of human enteral adenoviruses in diarrhea specimens collected at Johns Hopkins University.

3) Pneumococci: A successful assay has been developed to type pneumococci and to quantitatively and accurately assess antipneumococcal antibody production in splectomized patients following vaccination with a polyvalent pneumococcal antigen preparation[2,3].

4) DNA: An assay has been developed to detect DNA at concentrations as low as 1 to 10 nanograms/ml using human serum collected from patients with active systemic lupus erythematosis. This assay is 100 to 1,000 times more sensitive than the routine methods currently used.

Significance to Biomedical Research and the Program of the Institute:

Rapid assay for a variety of antigens and antibodies has broad applicability to both clinical and research areas. The tests currently being developed will speed up, simplify, and reduce the cost of detecting infections. At the same time, they will permit clinical decisions regarding therapy to be soundly based at an earlier time in the course of an illness than is currently practiced.

Proposed Course:

Once the ELISA tests are fully developed and their sensitivity and specificity defined, they will be examined prospectively with patient material. Their ability to correctly identify infectious agents will be tested by comparison with the results of traditional methods. Assays for viral agents will also be applied to routine testing of research materials generated in the study of virus pathogenesis and treatment.

Publications:

1. Berg, R.A., S.I. Rennard, B.R. Murphy, R.H. Yolken, R. Dolin, S.E. Straus: New enzyme immunoassays for measurement of influenza A/Victoria/3/75 virus in nasal washes. Lancet 1:851-853, 1980.

2. Rennard, S.I., R. Berg, G.R. Martin, J.M. Foidart, P.G. Robey: Enzyme-Linked Immunoassay (ELISA) for Connective Tissue Components. Anal. Biochem. 104:205-214, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00189-02 LCI

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Clinical and Biochemical Studies of Human Enteral Adenovirus Infections

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S.E. Straus	Senior Investigator Medical Virology Section	LCI NIAID
Other:	H. Takiff	Clinical Associate MVS	LCI NIAID

COOPERATING UNITS (if any)
A.Z. Kapikian (LID, NIAID), H.S. Ginsberg (Columbia University), J. Rose (LBV, NIAID), R. Yolken (Johns Hopkins), J. Esparza (Caracas, Venezuela)

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SECTION
Medical Virology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Enteral adenoviruses are a recently discovered group of agents which cause gastroenteritis in children. They differ from all other known human adenoviruses in their inability to be propagated in tissue culture. During the past year we have established channels to obtain specimens containing these unusual agents. Stool specimens have been characterized by enzyme immunoassay, electronmicroscopy, and attempted propagation in tissue culture. These agents have been studied to determine the biological basis for their inability to grow in tissue culture. Immunofluorescent microscopy, complementation experiments utilizing a variety of other viruses, sucrose gradient and DNA hybridization analysis have been performed to probe the biochemical events in enteral adenoviral infection. In summary we have learned that these agents induce an abortive infection which is abruptly terminated at a very early step of the virus growth cycle.

Project Description:

Objectives:

1) To define the clinical spectrum of illness induced by enteral adenoviruses in both naturally occurring and experimental infections.

2) To define the biochemical properties of these adenoviruses and their relation to other respiratory adenoviruses.

3) To develop methods of promoting the growth of these agents in tissue culture so that further studies will be simplified.

Methods Employed:

1) Source of viral agents: Stool specimens believed to contain adenoviruses have been obtained in collaborative efforts from natural infections in infants in Washington D.C., Baltimore, Bangladesh, and Venezuela.

2) Identification of Agents: Presumptive agents are characterized by enzyme immunoassays performed here or in Baltimore utilizing antisera developed in this laboratory. Electronmicroscopy is performed by Dr. Kapikian to identify and quantitate the adenovirus content of specimens. The specimens are then innoculated and blind passaged in primary and continuous human cell lines which are then examined for adenoviral cytopathic effects over a four to six week period. Agents which by electronmicroscopy have the structure of adenoviruses, react as such immunologically and fail to propagate are considered candidate enteral agents.

3) In vitro tissue culture studies: Numerous primary and continuous human and simian, adult and fetal tissues have been innoculated in microculture assays and examined for cytopathic effect and production of late viral proteins by immunofluorescence microscopy. In these studies enteral adenoviruses failed to replicate. In complementation studies the ability of other viruses to complement the replication of enteral agents was explored. Unlike respiratory adenoviruses these agents are not helped by SV40 in monkey cells. Wild type adenoviruses types 2 and 5 do not assist the replication in simian or human cells. Moreover the growth of the wild type respiratory viruses is not inhibited by coinfection with the enteral agents. Complementation does not occur in coinfections with a variety of early or late temperature sensitive mutants of adenovirus 5 provided by Harold Ginsberg of Columbia University. An adenovirus transformed cell line (293) does not support the growth of enteral viruses either. Unlike all other known adenoviruses the enteral agents do not help the defective parvovirus AAV.

Hamster adenovirus type 2 T antigen sera was used in immunofluorescence experiments to determine whether enteral adenoviruses successfully complete early steps in virus replication. The agents examined to date appear to synthesize normal amounts of nuclear T-antigen.

4) Biochemical Studies: The above studies established that enteral adenoviruses are capable of entering cells, uncoating, transporting their genomes to the cell nucleus, and successfully transcribing and translating early messager RNA species. At some point thereafter, the replicative cycle is aborted and detectable late antigen or progeny virus are synthesized. To further delineate the point at which viral replication ceases we have performed Hirt extractions, sucrose gradient analysis, and DNA-DNA hybridizations. Within the limits of sensitivity of the systems employed these studies concluded that in *in vitro* culture systems enteral adenoviruses synthesized less than 0.5% of the expected amount of DNA.

Experiments described thus far indicate that enteral adenoviruses spontaneously cease replication at an extremely earlier stage in the growth cycle. Because helper function for AAV is not provided, and in preliminary experiments with Dr. James Rose there is no evidence of cell transformation, we conclude that only a very few early genes are being successfully transcribed and translated in *in vitro* infections with human enteral adenoviruses.

5) Clinical Studies: Using antiadenovirus sera prepared in this laboratory Dr. Robert Yolken of the Department of Pediatrics at Johns Hopkins University has established an enzyme immunoassay to detect adenoviruses in stool samples. He is currently and prospectively examining stools of children seen in the out-patient clinic at Johns Hopkins. These studies will provide basic epidemiologic data regarding the importance of these agents in that community as well as provide additional specimens for further basic science and future clinical studies.

Major Findings:

To date we have established sources for enteral adenovirus specimens, developed the means of unequivocally identifying the agents and have completed the first detailed biochemical studies of these viruses. The data obtained to date indicates that as with the other major viral diarrhea agents (rotaviruses, parvovirus-like agents) the enteral adenoviruses do not grow in tissue culture. Our biochemical studies have probed the mechanism of restriction of virus replication in tissue culture. Similar studies have not been performed with the other major viral diarrhea agents. Because the biology of adenoviruses is extremely well defined compared to that of the other diarrhea agents it is possible that what is learned about the replication of human diarrhea-producing adenoviruses will bear importantly on the understanding of the other viral diarrhea agents.

Proposed Course:

During the next year we propose to characterize the genome structure of these viruses by *in vitro* labelling and restriction endonuclease analysis, and to define the major virion coat proteins by *in vitro* labeling and SDS-PAGE. We will use these data as the basis for characterizing a series of isolates from various diarrhea infections around the world to attempt to ascertain whether there are multiple types of enteral adenoviruses or whether there is only one family of such agents. Further *in vitro* studies will be performed to determine which early viral proteins are being synthesized. We will attempt to establish whether virus can be propagated in primary human intestinal organ culture.

As we obtain larger volumes of virus containing specimens we will initiate safety testing and some material will be utilized in monkey challenge experiments and then hopefully in human volunteer trials. These experiments will permit detailed investigations of viral pathogenesis in host responses to virus infection.

Publications:

1. Flewett, T.H., A.S. Bryden, H. Davies, C.A. Morris: Epidemic viral enteritis in a long-stay children's ward. Lancet 1:4, 1975.
2. Dolin, R.: Viral infections of the gastrointestinal tract. In G.J. Galasso, et al. (eds.) Antiviral Agents and Viral Diseases. Raven Press, New York, NY, 1979, p. 289.
3. Wadell, G.: Classification of human adenoviruses by SDS-polyacrylamide gel electrophoresis of structural polypeptides. Intervirology 11:47, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00192-02 LCI

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Studies on Immediate Hypersensitivity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D.D. Metcalfe	Senior Clinical Investigator	LCI	NIAID
Other:	A. Saavedra Delgado	Medical Officer	LCI	NIAID

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Clinical Investigation

SECTION
Allergic Diseases Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2 2/12	1 2/12	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Proteoglycans have further been identified and characterized in cells participating in allergic reactions. Human eosinophils have been shown to contain a highly charged proteoglycan chondroitin 4-sulfate. The proteoglycan heparins of the rat mast cell have been examined to determine sub-group characteristics which have functional implications.

Preparations are underway to develop an animal model of food hypersensitivity. Preliminary experiments are designed to correlate gastrointestinal mast cell number with histamine content and further, to attempt to release this histamine both in vivo and in vitro.

Development of protocols to study human mast cells and their pathophysiologic function in mastocytosis and cold urticaria.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

To identify and characterize the proteoglycans synthesized, stored, and released by cells involved in immediate hypersensitivity, and to determine the biologic function of these molecules. To develop methods to study the pathophyiologic role of human mast cells in hypersensitivity states, with special emphasis on the gastrointestinal system.

Methods Employed:

1. Studies into proteoglycan structure and function: Proteoglycans of both eosinophils and mast cells are radiolabeled *in vitro* using radioactive molecules selectively incorporated into specific structural features of the proteoglycan molecule. These proteoglycans are then extracted and purified using a combination of ion-exchange chromatography and gel filtration. The structure of the final molecule is further elucidated by chromatographic techniques and by the use of selective polysaccharidases. The same techniques may be applied to the preparation of quantities of these molecules for the *in vitro* examination of their biologic function.

2. Studies on the pathophysiologic role of the mast cell: Two protocol studies have been approved during the past year which allow the study of human mast cells. In the first protocol, individuals with cold urticaria have an extremity challenged with ice water. This results in the release of mast cell substances which can be collected by venipuncture and subsequent identified and characterized as to a possible pathophysiologic role. In the second protocol, patients with mastocytosis who have multiple organ systems infiltrated with mast cells are examined as to their response to mediations which affect mast cell degranulation or which block the action of mast cell derived mediators (histamine).

Efforts are underway to develop an animal model of food hypersensitivity. To date, stains specific for the gastrointestinal mast cell and a radioenzyme assay for histamine have been established in the laboratory. Preliminary attempts to degranulate gastrointestinal mast cells *in vitro* are underway.

Major Findings:

During the past year, the characterization of human eosinophil proteoglycan has been completed. This eosinophil proteoglycan was shown to be primarily chondroitin 4-sulfate. The intact proteoglycan has a molecular weight of approximately 60,000, while individual glycosaminoglycan side chains are 20,000 M.W. The intact proteoglycan chondroitin 4-sulfate behaves on DEAE cellulose chromatography in a similar fashion to glycosaminoglycan heparin indicating its strong charge. This high charge would allow this proteoglycan chondroitin 4-sulfate to bind to other eosinophil constituents of opposite charge such as major basic protein. Thus, chondroitin 4-sulfate may play a role in eosinophil mediator storage. The study of the size polydispersity of rat mast cell heparin has also been completed. This proteoglycan heparin has been shown to consist of a family of related molecules, all of a family of which have a similar protein core and similar glycosaminoglycan side chains. The polydispersity is due to a variable number of side chains on the protein core. The study of the biologic function of this molecule must take this finding into account by the careful definition of the particular set molecules to be studied, as function will vary with differing molecular structure.

Protocols on cold urticaria and mastocytosis, as well as studied on gastrointestinal hypsersensitivity are recent and have not yet generated sufficient data for analysis.

Significance to Biomedical Research

The isolation, identification, and characterization of proteoglycans in those cells involved in immediate hypersensitivity contributes to the understanding of the physiology of the reaction itself. By virtue of their unique characteristics including large size, and high negative charge, proteoglycans appear to have the capacity to not only function in the intracellular storage of inflammatory mediators, but to influence events that follow mast cell degranulation. The development of protocols to examine human mast cell function in cold urticaria and mastocytosis will allow observations made on mast cell function *in vitro* to be applied to human mast cell mediated disease. Specifically in mastocytosis, the hope is to determine the best method of treatment in this mast cell disorder. The extension of mast cell studies into gastrointestinal hypersensitivity will directly lead to a detailed examination of the complex area of food allergy.

Proposed Course:

We intend to complete the characterization of proteoglycans of interest, and to begin to study the possible biologic role of these molecules. Protocols on cold urticaria and mastocytosis recently approved will be implemented. The development of an animal model for gastrointestinal hypersensitivity will be accompanied by the submission of a basic clinical study into food allergy.

Publications:

1. Metcalfe, D.D., L.M. Corash, and M. Kaliner: Human platelet arylsulfatases: Identification and capacity to destroy SRS-A. *Immunol.*, 37:723-729, 1979.

2. Metcalfe, D.D., and K.F. Austen: The structure and function of intracellular proteoglycans. In: *Monographs in Allergy* - "New Approaches to the Management of Allergic Diseases," 12th Symposium of the Collegium Internationale Allergologicum, New Orleans, La., Sept. 17-29, 1978, 14:236-248, 1979.

3. Metcalfe, D.D., R.A. Lewis, J.E. Silbert, R.D. Rosenberg, S.I. Wasserman, and K.F. Austen: Isolation and characterization of heparin from human lung., *J. Clin. Invest.*, 64:1537-1543, 1979.

4. Metcalfe, D.D., S.I. Wasserman, and K.F. Austen: Isolation and characterization of sulfated mucopolysaccharides from rat basophil leukemia (RBL-1) cells., Biochem., J., 185:367-372, 1980.

5. Metcalfe, D.D., N.A. Soter, S.I. Wasserman, and K.F. Austen: Identification of sulfated mucopolysaccharides including heparin in the lesional skin of a patient with mastocytosis., J. Invest. Derm., 74:210-215, 1980.

6. Shelhamer, J.H., D.D. Metcalfe, L.J. Smith, and M. Kaliner: Abnormal beta adrenergic responsiveness in allergic subjects: Analysis of iso-proterenol-induced cardiovascular and plasma cyclic AMP responses., J. Allergy Clin. Immunol., 66:61, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00202-01 LCI

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Pneumococci and Other Encapsulated Bacteria

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Stephen Hosea	Senior Investigator	LCI, NIAID
OTHER:	E. Brown	Clinical Associate	LCI, NIAID
	M. Frank	Head, Clinical Immunology Section and Chief, LCI	LCI, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Clinical Investigation

SECTION
Clinical Immunology Section

INSTITUTE AND LOCATION
NIAID, Bethesda, MD 20205

TOTAL MANYEARS: 2 8/12	PROFESSIONAL: 1 8/12	OTHER: 1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The critical role of Complement in host defense against experimental bacterial infections by Streptococcus pneumoniae has been documented. The alternative pathway of complement activation provides the primary host defense in nonimmune animals, whereas, the classical pathway is necessary for optimal effect of pneumococcal antibodies. Additional studies have concerned the role of complement in mediating certain aspects of sepsis, such as the respiratory distress syndrome, and the role of the spleen in clearance of pneumococcal bacteremia.

Project Description:

The general goals of this have been to study the role of complement and antibody in the prevention and host defense against pneumococcal infections.

Objectives:

1. To determine the contribution of the classical versus alternative pathway of complement activation in animals and man.

2. Assess the role of the classical versus alternative pathway in the interaction with anti-pneumococcal antibodies.

3. To develop a sensitive assay for pneumococcal antibodies.

4. To understand the role of immunization with either polysaccharide or whole organisms in the host immunologic response. To characterize the antibody response and the biological activities of the various classes of antibody.

5. To study the interaction of complement components with the reticuloendothelial system.

6. To understand the role of the spleen in host defense against pneumococcal bacteremia.

7. To appreciate the significance of cleavage products of complement activation in mediating the morbid consequences of septicemia.

Methods Employed:

There are multiple techniques used. Basic to all *in vivo* studies is quantitative blood culturing after intravenous pneumococcal injection. Techniques for biological radiolabelling of pneumococci with ^{125}I, ^{75}Se and ^{59}Fe have been developed for the study of organ localization of bacteria. Antibody of different classes has been raised to pneumococci in mice, rabbits, and guinea pigs. Cobra venom factor (CVF) is used to deplete the alternate pathway for *in vivo* and *in vitro* studies, and C4 deficient (C4D) guinea pigs and serum are used to study the alternative complement pathway in isolation. Standard techniques of complement titer determination are employed, and purified complement components are obtained from Dr. Carl Hammer. An ELISA assay for determination of antibody to type specific polysaccharide or to whole pneumococci has been developed. Heated RBC, IgG and IgM coated RBC are used to ascertain the status of the hepatic and splenic RES.

Major Findings:

Pneumococcal bacteremia has two phases, a phase of exponential clearance of organisms from the blood, and a phase of recrudescence of organisms. If the animal survives there is a third phase, the phase of resterilization of the blood stream. There are differences in the kinetics of these phases when different types of pneumococci are employed. The survival of the animal is absolutely dependent on the presence of complement, and the beneficial effects of active immunization are mediated entirely by complement. Normal animals localize most of the bacteria to the liver and in C4D or CVF treated animals more organisms localize to the spleen. In actively immunized animals, the maximum number of organisms localize to the liver. Splenectomy of guinea pigs induces a defect in bacterial clearance which is corrected by immunization but not corrected by intraperitoneal injection of spleen cell suspension.

We have developed an ELISA assay for detection of pneumococcal antibodies. We have shown that splenectomized individuals do not respond as well to our pneumococcal polysaccharide vaccine as normals. In addition, human antibodies to pneumococcal polysaccharide do not fix complement. However, our animal studies suggest that complement fixation is a critical amplification mechanism for host defense.

Significance to Biomedical Research:

Application of the principles learned from the above studies may allow better understanding of the toxemia and persistent early mortality of pneumococcal infections. More appropriate and improved therapeutic manipulations often follow better understanding of a disease entity.

Proposed Course:

Our objectives at this time are:

1. To understand the role of various serum proteins, particularly antibody of different classes and C3b in the removal from the blood and organ localization of pneumococci. We are also interested in exploring the importance in our system of fibronectin and C-reactive protein, both thought to have clearance functions in other systems. We also will explore their interaction in the complement.

2. We would like to understand the recrudescence phase of pneumococcal bacteremia, and intend to study the state of the RES during this period

3. Attempts to reconstitute splenic activity by reimplantation of spleen fragments or injection of spleen cell culture supernatants are ongoing.

4. To understand the observed differences between pneumococcal types in clearance, we will study differences in growth kinetics of the organisms in serum and broth systems, as well as explore the effects of pneumococcal polysaccharide on in vivo and in vitro opsonization systems. We will also make use of radiolabelled complement components to explore the possibility that different organisms activate and/or inactivate complement in the differing efficiencies.

5. To understand the mechanisms of protection against human pneumococcal disease we are doing experiments to explore the interaction of human pneumococcal antibody with complement. In addition, we are using the ELISA assay to determine the kinetics of IgM and IgG production after vaccination with mono and polyvalent vaccines in normal volunteers, and after polyvalent vaccine administration in various highly susceptible patient groups.

6. In vitro studies will center around opsonization of bacteria by C3b and its control by β1H and the C3b inactivator. The key role of type specific polysaccharide in virulence will also be evaluated for its effect on opsonization and phagocytosis.

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3. Hosea, S.W., Brown, E.J., Hammer, C.H., Frank, M.M.: The Role of Complement in a Model of the Adult Respiratory Distress Syndrome. J. Clin. Invest. In press.

4. Hosea, S.W., Brown, E.J., Frank, M.M.: The Critical Role of Complement in Experimental Pneumococcal Sepsis. J. Infect. Dis. In Press.

LABORATORY OF IMMUNOGENETICS
1980 Annual Report
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Annual Report of the Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, NIH, October 1, 1979 - September 30, 1980

Summary Report

The Laboratory of Immunogenetics attempts to bring modern techniques in protein structural chemistry, immunology and molecular biology to bear on fundamental questions of immunogenetics. Studies in the past year have concerned immunoglobulin allotypes and genes encoding immunoglobulins with emphasis on regulatory processes involved in gene expression. These studies have utilized latent allotypes and rabbit-mouse hybridomas. Structural studies have been concerned with membrane antigens encoded by the murine major histocompatibility complex and have included completion of the primary structure of one antigen and completion of partial structures for a number of others for comparison purposes. Research into techniques for protein microsequence analysis have emphasized gas chromatographic mass spectrometric methods and an enzymatic procedure for degradation of proteins into dipeptides beginning at the carboxyl terminal has been described. In addition, research continues on the preparation and use of hybridoma antibodies directed against cell surface molecules, as well as basic research into the formation of hybrids, especially those between cells from animals of different species.

New staff members in LIG include: Dr. Susan Jackson, from the University of Alabama at Birmingham; Dr. Gerald Marti, from Northwestern University; Dr. Claudia Quijano, from Bogota, Columbia and Dr. Mei-Chang Kuo from the University of Wisconsin. In addition to the permanent staff members and fellows, the laboratory has two employees under the stay-in-school program and eight students from all parts of the country have joined us during the summer for work and study.

Research Accomplishments

Rabbit Histocompatibility Antigens. Current studies on rabbit histocompatibility antigens involve procedures aimed at obtaining amounts of antigen large enough to do amino acid sequence studies without recourse to radiolabeled preparations. These studies involve growth of large numbers of (10^{10}) cells in order to obtain sufficient amounts of protein. The fractionation of the mixtures has employed several different kinds of lectins (Wilkinson) and have succeeded in fractionating the population of cell surface molecules. In addition, mouse hybridoma antibodies are being prepared against the various subfractions of the rabbit cells and these will be used in future isolation studies.

Mass Spectrometry and Sequence Analysis of Polypeptides. Mass spectrometric techniques are being utilized for structural analysis of peptides and proteins. It our aim to develop and apply rapid and sensitive methodology to molecules of immunologic interest such as immunoglobulins, membrane antigens, and receptors. In the past year, it has been possible to develop a method whereby polypeptides are degraded from the C-terminus with dipeptidyl carboxypeptidase (DCP). The dipeptides obtained by DCP digest can be identified by methods used for those obtained by amino terminal digests using amino peptidase enzymes (Krutzsch).

Primary Structural Analysis of Murine Transplantation Antigens. The genes in the mouse H-2 major histocompatibility complex determine a number of functions which are associated with immune recognition and reaction. Our laboratory has concerned itself with those genes encoding major histocompatibility antigens which are encoded at the K, D, and L loci in the murine MHC. The entire 340 amino acid structure of the K^b antigen has been nearly completed (Coligan) and progress is being made toward completion of the D antigen from the b haplotype (Maloy, Coligan). In addition, studies have concentrated on molecules of the d haplotype (Kimball, Coligan) and recent results indicate that in the D region of the this haplotype, at least three molecules are encoded. These have been designated as D, L, and D, respectively. Structural studies will concentrate on determination of primary structures for the three molecules of the D region complex.

Studies have begun on structural comparisons of different human histocompatibility antigens that are identical by serologic analysis, but have differences recognized in viral restriction assays. This work will involve the use of conventional human alloantisera, as well as the development of hybridoma antibodies against private specificities of human histocompatibility antigens (Marti). Structural comparisons will be by primary sequence analysis, as well as by peptide mapping techniques which will utilize high pressure liquid chromatography.

Genetic Studies on Rabbit Immunoglobulin. In this area work has concentrated on characterization of the products secreted by rabbit-mouse hybridoma cells. These cells secrete rabbit immunoglobulin chains, some of which have anomalous structures (Dreher). Results obtained to date indicate that although both H or L chains may be normal by structural and serologic criteria, unusual modes of covalent association are possible. In one instance, covalent association has been discovered between a rabbit H chain and a mouse L chain where the structure appears to be comprised of two H and one L chain. In addition, characteristics of the cells secreting the molecules are being examined with a view to obtaining appropriate mutants to do further fusion studies between various hybridomas in an attempt to make complete rabbit immunoglobulin antibodies and other products (Sogn).

Carbohydrate Antigens of Microbial Cell Walls. Collaborative studies on the structure of the group specific carbohydrate isolated from the group B streptococcus have shown that this complex polysaccharide contains a large amount of glucitol, an alditol which is present in all strains of group B but no other hemolytic streptococcus. Studies are in progress to determine whether the group B polysaccharide has the same backbone common to the group A, A-variant and C polysaccharides (Coligan).

Nonallelic Expression of Genes Encoding Rabbit Immunoglobulins. The notion that rabbit immunoglobulin allotypes are inherited as autosomal alleles has been challenged by observations of low concentrations of allotypes in various samples of rabbit immunoglobulin and antibody. Research efforts are focused on attempts to cause *in vitro* production of latent allotypes (Jackson) and to obtain fusion products between latent allotype producers and mouse myeloma cells (Sogn).

Another approach to the latent allotype problem concerns the use of hybridoma cells as sources for messenger RNA which is allotype specific. Progress is being made in obtaining translation products from these mRNA samples and when they are of sufficient purity, cDNA will be produced in order to prepare probes that can determine the number of allotypes present in an individual rabbit genome.

Monoclonal Antibodies Directed Against Cell Surface Proteins. Monoclonal antibodies have been prepared by hybridoma techniques in the past year. Several of these antibodies are being used in structural studies such as those directed against the H-2 antigen 31 (K^d) and others directed against the rabbit T cell tumor line RL-5 (Sogn). Monoclonal antibodies are also being prepared against simple proteins such as ovalbumin and insulin (Folks). These proteins have been prepared in order to provide material for studies of idiotypy.

Immunoregulation of T Lymphocytes. An anti-idiotype antisera was used to demonstrate cross-reactivity between surface T cell receptors for soluble antigens and a hybridoma antibody directed against the same antigen. Two other hybridoma anti-ovalbumin antibodies are being used as immunogens in goats in order to produce additional anti-idiotype sera to extend these studies. In addition, splenic T cells from mice primed with blast T cells having specificity against soluble protein antigens are responsive in culture to mouse serum anti-ovalbumin (Folks). These studies will continue to probe similarities and differences between receptors for antigens contained on T cells and those present on antibodies and will use hybridoma antibodies as the source of idiotypic antisera (Sogn).

Honors and Awards

Dr. Kindt was invited to serve on the editorial boards for the *Journal of Immunogenetics*, and for *Current Topics in Molecular Immunology* and continues to serve on the boards of the *Journal of Experimental Medicine*, *Molecular Immunology*, *Proceedings for the Society for Experimental Biology and Medicine*, and *Immunology*. He serves on the program committee of the American Association of Immunologists and as block chairman organized a series of minisymposia on Immunologulin and Lymphocyte Receptors, which was held at the annual meeting in Anaheim, California. Results of laboratory research were presented at seminars at the Tissue Culture Association Meeting, at Georgetown University, at the New York State Public Health Service in Albany, New York and at the University of Texas in Dallas. Invited presentations were also made at the International Symposium on Inflammation which was held in Bogota, Colombia, and at a conference on Control of Cell Division held in Keystone, Colorado, as well as at the International Conference on Peptides and Proteins Sequence and Synthesis in Bethesda, Maryland. Dr. Kindt will organize and chair a session at the International Congress of Immunology in Paris. He has also served as co-chairperson for a section on Membrane Antigen and Cellular Interactions which was part of the Immunology Study Group and has begun participation in programs aimed at further cooperation between scientists in the United States and the Peoples Republic of China.

Dr. Coligan has presented data from his section at the American Association of Immunology meeting in Anaheim, California and at the International Congress of Immunology in Paris. Dr. Krutzsch read a paper describing his work at the International Congress on Proteins and Peptides our Sequence and Synthesis. In addition, he gave seminars on his work at the Bureau of Biologics in Bethesda, and at the University of Alabama in Birmingham. Dr. John Sogn served on the Immunology Study Group in the Section on Hybridomas.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00166-03 LIG

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Chemical Characterization of Rabbit Histocompatibility Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. Thomas J Kindt	Chief, LIG	LIG	NIAID
Others:	Dr. Edward S. Kimball	Staff Fellow	LIG	NIAID
	Dr. J. Michael Wilkinson	Expert	LIG	NIAID
	Dr. Claudia Quijano	Visiting Fellow	LIG	NIAID

COOPERATING UNITS (if any)

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Laboratory of Immunogenetics

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Immunogenetics Research Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.3	0.2

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XX

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The surface glycoproteins of the virally transformed rabbit T lymphocyte line, RL-5, have been studied by detergent lysis of metabolically radiolabeled cells followed by affinity chromatography on a variety of insolubilized lectins. Fractionation of the glycoprotein pool obtained from lentil lectin (LcH) by chromatography on Ricinus communis agglutinin (RCA) gives rise to two fractions. The first, which is not bound to the RCA column, contains mainly the major histocompatibility antigen, RL-A, as judged by gel electrophoresis, while the material which binds to the RCA column contains glycoproteins with apparent molecular weights greater than 70,000 daltons. This separation has been scaled up by the use of larger numbers of cells (10^{10} or more) in order to obtain sufficient amounts of protein for amino acid sequence determination and for immunization of mice in order to raise hybridoma antibodies directed against the various cell surface glycoproteins.

Chemical Characterization of Rabbit Histocompatibility Antigens

Determination of the N-terminal sequence of the RL-A molecules obtained from the RL-5 cell line by use of chromatography on an anti-β_2 microglobulin affinity adsorbent gave a single amino acid sequence. The present investigation was undertaken with a view to preparing the RL-A molecules by a different route, either by lectin chromatography or by the use of hybridoma antibodies, if available, in order to assess whether the apparent homogeneity was due to a real restriction of expression in the cell line (or in the animal) or was an artifact of the lectin and anti-β_2 microglobulin chromatography.

Initial results have shown that chromatography of solubilized membrane material on LCH-Sepharose followed by RCA-Sepharose gives a significant purification of the RL-A population of the cell. Glycoproteins of molecular weight greater than 70,000 daltons are bound specifically to the RCA-Sepharose column while the material passing through unretarded contains largely RL-A molecules. Chromatography of material that did not bind to LCH-Sepharose on wheat germ agglutinin (WGA)-Sepharose led to the isolation of a further glycoprotein pool. It is not yet clear whether this contains any further RL-A antigen molecules. This preparation is now being scaled up in order to obtain sufficient material for further characterization.

Attempts have been made to isolate material from the LCH glycoprotein pool with two different hybridoma antibodies. The first, the product of a rat-mouse hybridoma (obtained from T. McKearn) is directed against rat AgB molecules, but appears to react in an indiscriminate manner with the histocompatibility antigens of other species. The particular antibody used, D4-37, binds to the plasma membrane of RL-5 cells, as judged by the use of a fluorescent anti-rat IgM antibody, but it has been impossible to find conditions under which this antibody will precipitate material from the solubilized membrane. The second hybridoma antibody, obtained by immunization of mice with whole RL-5 cells, also reacts with the RL-5 cell membrane and attempts to use this to precipitate material from the glycoprotein pool are continuing.

Further biochemical characterization will be carried out to assess the homogeneity of the material. Two dimensional gel electrophoresis will be carried out together with peptide mapping. Material obtained from normal and inbred rabbit spleen cells treated by the same procedure will give information concerning the restriction in expression of RL-A molecules on RL-5 cells.

It is further intended to scale up the preparation of RL-A by use of material from about 10^{11} RL-5 cells. This should give sufficient protein for sequence analysis by the dipeptidyl peptidase GC/MS technique without having to resort to radiosequence techniques.

There are at present few well defined antisera available to the surface antigens of rabbit lymphocytes. It is intended to use the fractions obtained from the lectin chromatography to raise hybridoma antibodies and it is hoped that this may provide a general method which may prove superior to immunization with whole cells.

Publications

Kindt, T.J., Coligan, J.E., Kimball, E.S., Ewenstein, B., Uehara, H., Martinko, J., and Nathenson, S.G.: Use of radiochemical techniques for primary structural analysis of mouse and rabbit histocompatibility antigens. In Bearn, A.G., and Choppin, P.W. (Eds.): Receptors and Human Diseases. New York, Josiah Macy, Jr. Foundation, pp. 80-97, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00167-03 LIG

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Mass Spectrometry and Sequence Analysis of Polypeptides

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. Henry C. Krutzsch	Expert	LIG NIAID
Others:	Dr. Thomas J. Kindt	Chief, LIG	LIG NIAID

COOPERATING UNITS (if any)
None

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In immunogenetic and other studies, a need exists for methods for amino acid sequence analysis of proteins that are rapid and sensitive. Techniques involving mass spectrometry (MS) appear to be a promising way for meeting these goals. A dipeptidyl peptidase/gas chromatography-mass spectroscopy (DP/GC-MS) approach has been developed. This method allows polypeptide sequences to be determined from either the N- or C-terminus of the polypeptide in nanomolar amounts. The method has been enhanced by the use of computer programs to simplify data handling. Another method for use of MS in these determinations involves direct introduction of suitably modified polypeptides of approximately ten residues or less.

The DP/GC-MS method is currently being utilized to elucidate the structure of the N-terminal 225 residue fragment resulting from cyanogen bromide cleavage of an a3 allotype homogeneous rabbit IgG heavy chain.

Mass Spectrometry and Sequence Analysis of Polypeptides

Mass spectrometry will be utilized for structural analysis of peptides and proteins. A major objective of this program is to develop and apply the rapid and sensitive GP/GC-MS method to molecules of immunologic interest, such as immunoglobulins, membrane antigens and receptors. Another objective is to increase the sensitivity and scope of the method, such that the method can approach the levels obtainable by radiochemical methods. This will include devising strategies for isolation and purification of small amounts of peptide, such as by HPLC.

Polypeptides to be analyzed by the DP/GC-MS method are digested into dipeptide fragments, either from the N-terminus with dipeptidyl aminopeptidase (DAP), or from the C-terminus with dipeptidyl carboxypeptidase (DCP). The dipeptides are then identified by GC-MS as their trimethylsilyl (TMS) derivatives. Two MS ions are necessary for identification, the sequence-determining ion resulting from loss of the C-terminal residue plus the N-terminal residue's carbonyl group, and the molecular weight-determining ion, resulting from loss of a silyl methyl group from the parent molecule. The peptide is then modified by addition or subtraction of one residue at the N- or C-terminus, respectively, and again digested into dipeptides. Combination of this set of dipeptides and those from the unmodified polypeptide allows reconstitution of the polypeptide sequence.

The studies concerned with the development of the DCP aspect of the method have been completed for the most part. Over twenty polypeptides, of both known and unknown sequence, have been successfully analyzed in amounts as low as 5 nmoles, with lower levels possible. The DCP used in these studies was Angiotensin coverting enzyme. Polypeptides with chain lengths as high as 49 residues and containing all types of amino acids were analyzed in this study. The modified polypeptide was obtained either by addition of a single Aba residue to the C-terminus or by subtraction of the C-terminal residue. Conditions determined for these operations allowed production of the modified polypeptide in yields of approximately 95% or greater. Tryptic peptides, because they terminate in a basic residue, are especially good candidates for DCP because modification of the polypeptide can be carried out with carboxypeptidase B, which selectively removes basic C-terminal residues. The use of DCP has the advantage over both the DAP and Edman methods of not requiring a free polypeptide N-terminus. The use of DCP allowed completion of the sequence of an IgG cyanogen bromide fragment, which had been analyzed up to the last eight residues by the Edman method. No further information could be obtained from the Edman procedure and attempts at analysis with carboxymonopeptidases gave ambiguous results. DCP has also been successfully applied to sequence analysis of several tryptic peptides from rabbit IgG a3 allotype heavy chain. This work is continuing.

The development work on three computer programs to facilitate the DP/GC-MS method has been completed. One of these is for assisting manual identification of TMS dipeptides from their mass spectra. Another of these allows automatic identification of TMS dipeptides from their computer-stored mass spectra. To do this, the program compares all ions in the mass spectra of appropriate intensities to a library of ions of masses specific for each dipeptide. If a match is found for both the sequence-determining ion and the molecular weight-determining ion, the dipeptide is printed out as a possible identification. Final identification is made from consideration of the accompanying selected raw data. A third program aligns the two sets of dipeptides from dipeptidyl peptidase digestion of the native and modified polypeptide into the polypeptide sequence.

The application of the DP/GC-MS method to the sequence analysis of the N-terminal 225 residue fragment resulting from cyanogen bromide cleavage of a homogeneous a3 allotype rabbit IgG heavy chain is in progress. Work in this area has been concentrated on obtaining and analyzing the tryptic fragments from this molecule. Because most of the fragments resulting from the initial restricted digestion (Arg only) are blocked at the N-terminus due to gluatmine-pyroglutamate conversion, the DCP aspect of the DP/GC-MS method should prove to be a useful asset.

Publications

Yarmush, M.L., Krutzsch, H.C., and Kindt, T.J. Amino acid sequence analysis of immunoglobulin light cains by gas chromatographic-mass spectrometric techniques: structural identity of latent b9 and nominal b9 molecules. Molecular Immunology 17: 319-326, 1980.

Krutzsch, H.C.: The determination of polypeptide amino acid sequences from the carboxyl terminus using angiotensin I converting enzyme. Biochemistry, in press, 1980.

Krutzsch, H.C.: Mass spectral strategies for polypeptide amino acid sequence analysis with dipeptidyl peptidases. In Schechter, A.N., and Liu, T.Y. (Eds.): The Chemical Synthesis and Sequencing of Peptides and Proteins. In press, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00168-03 LIG

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Structure of Rabbit Immunoglobulin and Antibody Heavy and Light Chains

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Dr. Thomas J. Kindt	Chief	LIG NIAID

COOPERATING UNITS (if any)

None

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SECTION
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TOTAL MANYEARS: 0	PROFESSIONAL: 0	OTHER: 0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been inactive in the past year. A publication of work completed in the previous year and stated as in press has been published.

Publications

Yarmush, M.L., Krutzsch, H.C., and Kindt, T.J. Amino acid sequence analysis of immunoglobulin light chains by gas chromatographic-mass spectrometric techniques: structural identity of latent b9 and nominal b9 molecules. Molecular Immunology 17: 319-326, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00169-03 LIG

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Primary Structural Analysis of Murine Transplantation Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. John E. Coligan	Research Microbiologist	LIG	NIAID
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	Dr. Frederick T. Gates	Staff Fellow	LIG	NIAID
	Dr. W. Lee Maloy	Staff Fellow	LIG	NIAID
	Dr. Edward S. Kimball	Staff Fellow	LIG	NIAID

COOPERATING UNITS (if any)

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National Cancer Institute, National Institutes of Health (Dr. David Sachs)
Merck Institute for Therapeutic Research (Dr. Ted Hansen)

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SECTION
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TOTAL MANYEARS: 5.3	PROFESSIONAL: 3.3	OTHER: 2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The genes in the mouse H-2 major histocompatibility complex (MHC) determine a diversity of functions associated with immune recognition and reaction. Three of these genes, H-2K, H-2D and H-2L encode antigens involved in allograft rejection and T-lymphocyte responses. The purpose of this work is to determine the primary structure of these molecules in order to better understand their function and mechanism of action. The determination of the amino acid sequence of the extracellular portion (~280 residues) of the H-2K^b MHC antigen has been completed and that of the transmembrane portion (~25 residues) and the intracellular portion (~35 residues) of this molecule are nearing completion. For comparative purposes, major portions of the primary structure of four other H-2 molecules (H-2D^b, K^d, L^d and D^d) have been completed and recent immunoprecipitation studies suggest the presence of a third H-2 molecule encoded at the D-end of the MHC which has tentatively been designated H-2O. The determination of the amino acid sequence of murine β_2m, the light chain associated with certain molecules encoded by the MHC has resulted in the detection of at least one variable position. Previous studies of this molecule isolated from humans and rabbits have indicated it to be nonpolymorphic.

PHS-6040
(Rev. 10-76)

Primary Structural Analyses of Murine Transplantation Antigens

A major objective of this research is to gain a better understanding of the molecular structure of membrane molecules with emphasis on those playing prominent roles in the function of lymphoid cells. Knowledge of the structures of these molecules will hopefully provide insight into how these molecules function, i.e. recognize extracellular signals, transmit these signals across the cell membrane, and how they maintain their orientation in the membrane. In addition, these structural studies should define evolutionary relationships among these molecules providing clues to the origin of these molecules and possible functional relationships among them.

For our studies to date, we have relied on radiochemical methodology. Tumor cell lines or spleen cells are labeled in short term culture with tritiated amino acids of high specific activity (^{35}S and ^{14}C amino acids are used for special applications). The cells are harvested and the membranes are solubilized with Nonidet P-40 and the glycoprotein and protein components are separated by lectin chromatography. Specific molecules are isolated from these fractions by immunoprecipitation with appropriate alloantisera or monoclonal antibodies. Biochemical properties of these molecules are determined utilizing classical protein chemistry techniques which have been adapted to the radiochemical approach. Peptides amenable to automated protein sequencing are obtained by CNBr digestion or enzymatic cleavage and are separated by gel-filtration chromatography, ion-exchange chromatography or high pressure liquid chromatography (HPLC). The radiolabeled peptides are sequenced with unlabeled carrier protein using standard sequencing programs on a Beckman amino acid sequencer. The radiolabeled PTH-amino acids are identified by co-chromatography on reverse phase HPLC with unlabeled PTH amino acid standards.

The determination of the amino acid sequence of the entire H-2K^b molecule (346 residues) is nearly complete; the NH_2-terminal 285 residues have been completely determined. The molecule can be conveniently divided into three regions of 90 residues and a fourth consisting of the COOH-terminal region of approximately 80 residues. The regions are designated N for NH_2-terminal (residues 1-90), C1 for the first disulfide loop (residues 91-180), C2 for the second disulfide loop (residues 181-270) and TM for the transmembrane portion (residues 271-347).

Region N contains the first carbohydrate moiety (position 86) and the portion of the molecule which exhibits the highest variability (positions 61-82) when H-2 and HLA molecules are compared. The overall homology of K^b compared to HLA-B7 and A2 is about 70% whereas the homology (positions 61-82) in this region is 35%.

Region C1, in addition to containing the first disulfide loop (Cys 101 - Cys 164), contains a second carbohydrate moiety (position 176). This carbohydrate moiety is absent in HLA molecules, which possess the carbohydrate at position 86, but does occur in all other H-2 molecules which have been examined (see below).

Region C2 contains the second disulfide loop and is the region which has homology to immunoglobulin constant domains and to β_2m. This region also contains a stretch of residues (positions 172-199) in which the homology to HLA-B7 is only 50%, which is significantly less than the overall homology of 70%. Since all murine molecules appear to have a carbohydrate moiety (position (176) attached in this region, whereas HLA molecules lack this carbohydrate, the amino acid sequence variation in this region may be related to the presence or absence of a carbohydrate attachment site.

The region designated TM can be divided into three subregions, an extracellular portion (residues 271 to 284), an intra-membrane portion (~residues 285-307), and an intracellular portion (~residues 308-346). The extracellular portion of this region contains the site(s) of papain cleavage which is responsible for the classic "papain fragment". In H-2K^b, we have determined that papain cleaves after the valine residue at position 281, whereas in the human, papain cleavage sites have been reported at positions 271 and 273. Positions 285-307 contain only hydrophobic amino acids residues. The intracellular portion initiates with the sequence Arg-Arg-Arg. This or a similar stretch of basic residues has been detected in other membrane molecules i.e. HLA and glycophorin, and may act as some sort of plug to keep such molecules anchored in the membrane. The remainder of the intracellular portion (~residues 313-346) has been the most difficult portion of the molecule to isolate and when isolated it has proven difficult to determine its sequence on the automated protein sequencer. Approximately 90% of the amino acid sequence of this region has been determined.

In order to gain more complete knowledge of the variability among H-2 molecules and to gain an understanding of the relationships among these molecules, the complete amino acid sequences for the major H-2 molecules of the b and d haplotypes are being determined. Initial results indicate that the gross structural properties appear to be the same in all the molecules i.e., all appear to possess two carbohydrate moieties in probably identical positions (86 and 176), all apparently possess two disulfide loops connecting the same positions (101-164; 203-259) and, of importance for structural studies, the Met (CNBr cleavage) residues are relatively invariant.

The determination of the amino acid sequence of these other H-2 molecules is at various stages of completion. They are as follows: (1) for the H-2D^b molecule 148 out of the NH$_2$-terminal 284 residues (52%) have been determined including the amino terminal 100 residues; (2) for the H-2K^d molecule, 110 out of 284 residues (39%) have been determined including 83 of the NH$_2$-terminal 100 residues; (3) for the H-2D^d molecule 178 out of 284 residues (63%) have been determined again with emphasis on the NH$_2$-terminal and, finally; (4) for the H-2L^d molecule 61 out of the 284 residues (21%) have been determined.

The overall homology to H-2K^b of H-2D^b, H-2K^d, H-2D^d, and H-2L^d for the partial amino acid sequences available is 87, 76, 87, and 78 percent respectively. Interestingly, the two D-end molecules D^b and D^d show higher homology to H-$2K^b$ thus far than does H-$2K^d$. K^d and D^d can be compared at 91 positions and show an homology of 78%. Comparison of D^b and D^d at the 126 positions available for comparison yields an homology of 86%. Thus, when one either examines overall homology or discrete regions of amino acid sequences which are available for comparison, there is no evidence of K-ness or D-ness. This agrees with previous peptide mapping studies.

In the partial amino acid sequences available for comparison among the H-2 molecules, no single region of variability can be discerned. The K^d molecule does show the same section of high variability (positions 61-82) when compared to K^b as do HLA-B7 and A2. The homology of H-$2K^d$, HLA-B7 and HLA-A2 to H-$2K^b$ is 52%, 36% and 36% respectively in this region. However, the variability of D^b and D^d when compared to K^b (83% and 80% homology, respectively) is about average in this region.

Sequential precipitations of labeled B10.AKM ($K^kD^qL^q$) spleen cells with various antisera to D^q region antigens have demonstrated that there are at least three distinct molecules defined by this antisera. One antiserum [(LP.RIII X B10.A) anti B10.AKM] (anti H-2.30) appears to react with a single molecule, most probably D^q. A hybridoma antibody, which was reactive with L^d but not D^d, appears to react with two D^q end molecules which we tentatively have designated D^q and L^q. A third reagent (AKR anti-AKR.M) appears to react with at least three D-end molecules tentatively designated D^q, L^q and O^q. The question as to whether this third D-end molecule is a member of the H-2 series of molecules or another molecule coded for by the 17th chromosome such as a member of the Qa series is being examined by structural studies. (Similar results were obtained with an AKR.M tumor cell line).

The complete primary structure of murine β_2m has been determined using radiosequencing methodology. Large amounts of radiolabeled β_2m are produced as a by-product during the isolation of H-$2K^b$, K^d and D^d (little β_2m is coprecipitated with the D^b and L^d heavy chains). The murine β_2m is 71% and 69% homologous to human and rabbit β_2m, respectively. Both the rabbit and murine molecules are one residue shorter than the 100 residue human molecule. The overall homology between human and murine β_2m is very similar to the overall homology between the HLA and H-2 heavy chains.

Most previous studies in other species have indicated that β_2m is nonpolymorphic. However, recently we have detected an amino acid sequence difference between murine β_2m molecules isolated from different sources. This difference is an Ala at position 85 of β_2m isolated in association with H-$2K^b$ from EL-4 cells versus an unknown amino acid substitution at this position for β_2m isolated in association with H-$2D^d$ from C14 tumor cells.

It is hoped that within the next year or two the primary structures for three additional H-2 molecules (K^d, D^d, and D^b) will be completed and that a significant additional percentage of the primary structure of the H-$2L^d$ molecule will be determined. This work should proceed relatively rapidly since most of the radiolabeled glycoproteins have already been prepared. In conjunction with these studies, peptide mapping and primary structural determinations will be undertaken to determine the interrelationship of H-2D^q, H-2L^q and the recently described H-20^q molecule. Previous serological studies have suggested an evolutionary relationship among H-2D^q, L^q and L^d. These studies will be extended to determine the primary structural relationship of the q haplotype molecules to H-2L^d.

Future studies on murine β_2m will focus on the recently discovered polymorphism at position 85. Studies are in progress to determine the extent of this polymorphism, i.e. is the polymorphism unique to a particular cell line being used, is it strain specific or are slightly different β_2m molecules associated with different membrane molecules in a given mouse strain. If a true genetic polymorphism is found, we will attempt to map the gene responsible for the synthesis of the molecule by using the appropriate recombinant inbred strains of mice. Either peptide mapping or amino acid sequence determinations of the appropriate tryptic peptide will serve to distinguish the polymorphic forms.

A major goal of our structural studies on the H-2 glycoproteins is to locate positions of structural changes in the H-2 molecules isolated from the series of H-2K and H-2D mutants of the b and d haplotypes. Strains of each K and D mutant series show strong histogenic reactivity *in vivo* and MLR and CML *in vitro* with the parents. In all of the H-2K^b mutants that have been examined, there appear to be only one or two amino acid differences between the H-2K molecules, produced by the parent and mutant strain. Further studies on the precise nature of the amino acid interchanges in these and other H-2K molecules of this mutant series will serve to localize the region of the molecule in which polymorphism can serve as a specific and effective signal for recognition by the immune system. Knowledge of this recognition is important since molecules coded for by the K and D loci of the major histocompatibility complex appear to play a major role in the stimulation and specificity of T-lymphocyte responses to virally induced and other cell surface antigens.

Publications

Kindt, T.J., Coligan, J.E., Kimball, E.S., Ewenstein, B., Uehara, H., Martinko, J., and Nathenson, S.G.: Use of radiochemical techniques for primary structural analysis of mouse and rabbit histocompatibility antigens. In Bearn, A.G., and Choppin, P.W. (Eds.): *Receptors and Human Diseases*. New York, Josiah Macy, Jr. Foundation, pp. 80-97.

Nathenson, S.G., Ewenstein, B.M., Uehara, H., Martinko, J.M., Coligan, J.E., and Kindt, T.J.: Recent studies on the structure of the H-2K^b glycoprotein and on the H-2K MHC mutants. In Ferrone, S., and Reisfeld, R. A. (Eds.): *Current Trends in Histocompatibility*. Plenum Press, in press.

Uehara, H., Ewenstein, B.M., Martinko, J.M., Nathenson, S.G., Coligan, J.E., and Kindt, T.J.: Primary structure of murine MHC alloantigens: amino acid sequence of the amino terminal 173 residues of the H-2K^b glycoprotein. Biochemistry 19: 306-315, 1980.

Coligan, J.E., Kindt, T.J., Nairn, R., Nathenson, S.G., Sachs, D.H., and Hansen, T.H.: Primary structural studies of an H-2L molecule confirm that it is a unique gene product with homology to H-2K and H-2D antigens. Proc. Natl. Acad. Sci., U.S.A. 77: 1134-1138, 1980.

Nairn, R., Nathenson, S.G., and Coligan, J.E.: Isolation, characterization and amino acid sequence studies of the cyanogen bromide fragments of the H-2D^d glycoprotein. Eur. J. Immunol., in press, 1980.

Kimball, E.S., Maloy, W.L., Martinko, J.M., Nathenson, S.G., and Coligan, J.E.: Structural studies on H-2D^b and H-2K^d molecules indicate that murine major histocompatibility *b* and *d* haplotype alloantigens share structural features. Molecular Immunol., in press, 1980.

Maloy, W.L., Hämmerling, G., Nathenson, S., and Coligan, J.E.: Comparison of alloantisera and hybridoma antibody for purification of the H-2D^b murine histocompatibility antigen and preliminary molecular characterization of this antigen. J. Immunol. Methods, in press, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00170-03 LIG

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Structural Studies on Precursors to Immunoglobulin Molecules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Others:	Dr. Frederick T. Gates	Staff Fellow	LIG	NIAID

COOPERATING UNITS (if any)

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LAB/BRANCH

Laboratory of Immunogenetics

SECTION

Immunogenetics Research Section

INSTITUTE AND LOCATION

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has been inactive in the past year; a publication of work completed in the previous year is in press.

Publications

Singer, H.H., Gates, F.T., Kindt, T.J., and Williamson, A.R.: Partial amino acid sequence of precursors of blocked heavy and light chains from a C3H mouse myeloma: Identification of a new V_H subgroup. Eur. J. Immunol., in press, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00171-03 LIG

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Genetic Studies on Rabbit Immunoglobulins and Other Serum Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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XX

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The principal study carried out in the past year has been the characterization of rabbit-mouse hybridoma cells secreting rabbit immunoglobulin chains. Secreted products of several cell lines have been isolated and structural characterization is in progress. Results obtained to date indicate that the rabbit secreted products (either H or L chain) are normal both structurally and serologically. An unusual mode of covalent association has been discovered between a rabbit H chain and a mouse L chain in one hybridoma product.

Several characteristics of the cells secreting these molecules have also been examined, particularly with a view to obtaining appropriate mutants for further fusion studies between various hybridomas.

Genetic Studies on Rabbit Immunoglobulins and Other Serum Proteins

Initial steps in the purification of the rabbit immunoglobulin a3 heavy chain produced by 7D2 has shown that the majority of ELISA-positive material for rabbit immunoglobulin, and RIA-positive material for a3 allotype, can be obtained by passing either ascites fluid or cell culture supernatant through DEAE-cellulose at low ionic strength and constant pH. These findings were confirmed and quantitative data concerning the levels of rabbit immunoglobulin were obtained using direct binding of radiolabeled DEAE-cellulose fractions to immunoadsorbents. Ascites fluid produced by injection 10^6 7D2 cells into nude mice was found to be 100 fold more concentrated with respect to rabbit immunoglobulin chain than that present in 5X concentrated tissue culture supernatant obtained at a maximum cell density of 2 X 10^6 cells/ml.

Rabbit immunoglobulin chain isolated from 7D2 ascites fluid by DEAE-cellulose ion exchange chromatography was radiolabeled and adsorbed onto anti-a3 Sepharose. Adsorbed material was eluted and analyzed by SDS-PAGE. Material isolated from 7D2 ascites fluid when analyzed under non-reducing conditions on 5% SDS gel gave a single peak with a molecular weight of 130,000. The same sample, when reduced gave two peaks on 10% SDS gels with molecular weights of 53,000 and 22,000. The 7D2 a3 positive product quantitatively binds to goat anti-mouse L chain antibodies but not to any anti-rabbit L chain antisera. The chain composition of the 130,000 mol. wt. material is still unknown. It may be possible that 2 rabbit H chains are joined in some fashion to a single mouse L chain.

The b4 light chain produced by cell line 12F2 has been isolated by procedures similar to those used for the 7D2 heavy chain. DEAE-cellulose chromatography of ascites fluid, followed by immunoadsorbent and/or gel filtration chromatography yields a fairly pure product with a normal L chain molecular weight of 22,000. A mouse L chain is secreted by 12F2 along with the rabbit L chain but the two chains are not covalently associated and do not appear to be noncovalently associated either. Studies on the isolation of the allotype-negative, ELISA-positive L chain from cell line 2C4 have just been initiated.

Propagation of an L chain producing hybridoma cell line (1D4P5) in ascites fluid produced sufficient material for preliminary structural analyses. Affinity purification on an anti-b5 Sepharose column yield approximately 1 mg of L chain which was used for sequence analysis. Twenty N-terminal residues were identified and these confirmed that this was a rabbit L chain. A minor sequence presumably from an internal cleavage (position 138) was characteristic of the b5 allotype.

Characterization and modification of rabbit-mouse hybridomas. While the fusion of mouse myeloma cells to rabbit splenocytes has proven a useable method to obtain at least a limited class of stable cell lines secreting rabbit products, the range of products available is limited by the chromosomal instability in that only one Ig chain has been stabilized per cell line and a great deal of work is required to obtain each line. These problems would be solved directly if a suitable rabbit myeloma cell were available. Unfortunately all attempts to obtain rabbit B cell lines have been unsuccessful in our hands. Viral transformation with a wide range of lymphotropic transforming viruses has not produced growth and the only known rabbit B cell tumor, the hereditary lymphosarcoma described by Fox at the Jackson Laboratory, could not be established in culture in experiments carried out in collaboration with Dr. George Moore at the Denver General Hospital.

The most promising approach to altering the available repertoire of rabbit-mouse hybrids is to use stabilized rabbit-mouse hybridomas as parental lines for further fusions. Fusions may be done between hybridomas and rabbit splenocytes or between two hybridomas. Such fusions would yield cells producing complete IgG molecules and they might yield cells secreting the desired products at much higher rates. All that is required is the introduction of appropriate drug on metabolic sensitivities into the stable hybridomas. Sensitivity to HAT medium, which is the only thing required for fusion to splenocytes, is immediately available because, when HAT medium is removed after hybridoma formation, the instability of rabbit chromosomal material assures a rapid and spontaneous loss of the rabbit HPRTase, with reversion to HAT sensitivity. Rabbit-mouse hybrids of H-chain-secreting and L-chain-secreting types have been cloned in 8-azaguanine to ensure HAT-sensitivity. Fusions using these clones will be done as soon as reagents are ready for a chain-specific ELISA screening assay. Fusions between two hybridomas require separate, complementary drug sensitivities in the fusion partners. In addition to HPRTase deficiency, APRTase and TKase deficiencies are easily introduced into these cells.

Another type of experiment which will be done is to fuse a hybridoma to cytoplasts obtained from a hybridoma secreting the same type of immunoglobulin chain (H or L) but of a different allotypic specificity. In some schemes for the regulation of allotype expression, cytoplasmic factors could be present which would induce synthesis of a new allotypic specificity upon cybrid formation. This will be tested using appropriate chloramphenicol-resistant mutants which have been cloned.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00172-03 LIG

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Carbohydrate and Glycoprotein Antigens of Microbial Cell Walls

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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0.1	0.1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies on the structure of the group-specific carbohydrate isolated from Group B streptococcus have shown that this complex polysaccharide contains a large amount of glucitol, an alditol present in all strains of Group B streptococcus analyzed but in no other hemolytic streptococci. Studies are in progress to determine whether the Group B polysaccharide has the same polyrhamnose backbone common to the Group A, A-variant and C polysaccharides. The composition of the Group B polysaccharide suggests this possibility.

During the course of these studies, a simple and highly sensitive gas chromatographic procedure was developed for analyzing the carbohydrate composition of whole bacterial cells. Chromatographic tracings of these carbohydrate compositions provide highly characteristic fingerprints of the hemolytic streptococci. The method has been used to analyze single colonies from a blood agar plate.

Carbohydrate and Glycoprotein Antigens of Microbial Cell Walls

Previous investigators have reported that the Group B-specific polysaccharide is composed of rhamnose, galactose and N-acetyl glucosamine, but they failed to account for a major portion of the dry weight of the polysaccharide. We found the composition of the group B-specific polysaccharide to be 45% rhamnose, 17% galactose, 25% N-acetylglucosamine plus an unidentified sugar. We have recently obtained evidence that this unidentified sugar is glucitol. This alditol is present in all types and strains of group B streptococcus analyzed but in no other hemolytic streptococci tested (Groups A, A-variant, C, G and F). Evidence indicates that the glucitol may contain phosphate groups. Analysis of the acetylated derivatives of the permethylated monosaccharides of the Group B-specific polysaccharide have shown the following sugars to be present: 1-linked rhamnose, 1,2-linked rhamnose, 1,3-linked rhamnose, 1,2,4-linked rhamnose, 1,3-linked galactose, and 1,3-linked N-acetyl glucosamine. The glucitol was not detected in this analysis. Current studies are focused on determining the structure of the Group B polysaccharide.

Hemolytic streptococci are routinely differentiated by the serological detection of the group-specific polysaccharide. This is usually done by mild acid extraction of the cell pellet from an overnight culture of the organism. The group-specific carbohydrate accounts for a major portion of the dry weight of the bacterial cell. We have developed a carbohydrate "finger printing" method based on the gas chromatographic separation of the trifluoroacetate derivatives of the component monosaccharides of streptococcal cells. This method which utilizes an electron capture detector is several thousand fold more sensitive and much more rapid than the serologic method for differentiating these pathogenic organisms. This method is sensitive enough to differentiate the serogroups of the strepococci by only analyzing a single bacterial colonies.

Publications

Coligan, J.E., Krause, R.M., and Kindt, T.J.: Use of Structurally Defined Streptococcal Carbohydrate Antigens in Studies of Rabbit Antibody Idiotypes. In Read, S. and Zabriskie, J. (Eds.): Streptococcal Disease and the Immune Response. Academic Press, New York, 1980, pp. 303-315.

Krause, R.M., and Coligan, J.E. Antibodies to streptococcal carbohydrate, -- substitutes for the myeloma proteins. Review of Infect. Dis.1: 904-910, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00173-03 LIG

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Nonallelic Expression of Genes Encoding Rabbit Immunoglobulins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS: 2.9	PROFESSIONAL: 1.8	OTHER: 1.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Rabbit immunoglobulin allotypes are antigenic determinants thought to be inherited as autosomal co-dominant alleles. This notion has been challenged by observations of low concentrations of allotypes not detected by qualitative tests or predicted by parental genotypes. In the past year, L chains with "latent" allotypes were isolated from the sera of pedigreed rabbits and shown by amino acid sequence analysis to be indistinguishable from the normal allotypes. Current research focus on hybridoma cells maintained in culture that synthesized rabbit immunoglobulin RNA will be isolated from these cell lines and cDNA probes will be prepared to carry out molecular biological studies in order to determine the basis for inheritance of latent allotypes. Other studies in this area have included clearance of normal versus latent allotypes in animals using doubly labelled material and studies on the linked expression of V_H markers on immunoglobulins expressing latent allotypes.

Nonallelic Expression of Genes Encoding Rabbit Immunoglobulins

Light chains with latent allotypes were isolated from the sera of pedigreed rabbits and shown by amino acid sequence analysis to be indistinquishable from sequences obtained from control samples of Ig with the allotype in question. In addition, studies have been completed concerning linkage between the expression of latent constant region and latent variable region allotypes. In these studies immunoglobulin or antibody molecules were isolated on the basis of a V region latent allotype and the C region allotype of these isolated samples was tested. It was found; that although 50% of the samples had latent C region allotypes, all samples expressed combinations of variable and constant region markers predicted from linkage data obtained in the rabbit families.

One approach to the latent allotype problem has involved the measurement of IgG clearance rates. These experiments utilized intravenous injection of rabbits with differentially labelled (^{125}I and ^{131}I) allotype matched and non-matched IgG samples. In no instance was the allotype matched IgG cleared faster than non-matched, although the converse was true in several instances. These data indicate that there is an *in vivo* recognition of allotypes which may be involved in possible regulatory mechanisms. In an attempt to further define the role of anti-allotype antibody in the regulation of allotype expression, rabbits were injected with anti-allotype antibody (directed against an allotype not present in the rabbit's nominal complement) over the course of several months and then immunized six months later with an antigen (streptococcal vaccine) to which they had previously responded. It was found that every animal subjected to the course of anti-allotype antibody injection produced large amounts of antistreptococcal antibody bearing the latent allotype against which the injected antibodies were directed. The amounts of latent allotype expressed by these animals following immunization with streptococcal vaccine was at least 10-fold higher than the average observed in a latent expressing animal.

It is our feeling that latent allotype expression in the rabbit offers a unique opportunity to study details of eukaryotic gene structure and regulation. However, the rabbit allotype model has not been exploited in the molecular biological studies of immunoglobulin genes that have recently provided exciting data for murine immunoglobulins. The primary reason for the lack of information concerning this model is the absence of suitable and reproducible sources of homogeneous antibodies and the nucleic acid precursors of these molecules that are necessary to construct the appropriate system for molecular biological studies. Attempts in our Laboratory to isolate messenger RNA from spleens of rabbits hyperimmunzed with antigens known to induce formation of homogeneous antibodies have met with limited or no success. Furthermore, searches for rabbit myelomas and attempts to transform rabbit B cells with viruses have likewise met with limited success.

In an attempt to circumvent this difficulty, research in the past years has been directed toward production of rabbit-mouse hybridoma cell lines that secrete rabbit immunoglobulin chains and that can provide a source for homogeneous immungobulins, DNA and messenger RNA. Our studies in this area have met with some success and research in this area has recently intensified. Data concerning initial preparation and are given below and other project reports contain descriptions of products produced and other experiments using these lines.

Standard cell fusion techniques involving the use of polyethylene glycol were used to generate hybrid cell lines from rabbit spleen cells and mouse myeloma cell lines. The hybrid cells obtained were selected for the secretion of rabbit immunoglobulin. This selection was carried out using an ELISA type assay designed to detect small amounts of rabbit immunoglobulin in the presence of large amounts of mouse immunoglobulin. Data indicated that rabbit spleen or lymph node cells gave reasonable fusion frequencies, whereas peripheral blood lymphocytes were not satisfactory for these experiments.

Approximately 189 cell lines retained the capacity to secrete rabbit immunoglobulins for periods long enough (~28 days) to obtain sufficient IgG material to do detailed allotypic analysis by radioimmune assay. These analyses revealed several interesting features of the cell lines. Of the 189 tested, only one produced an intact Ig molecule with antibody activity; one hundred and forty-three lines produced only rabbit L chains, 36 produced rabbit H chains. Nine of the cell lines were positive in the ELISA assay, but gave negative results in tests for group a and group b allotypes. It is assumed that these cell lines are secreting either H chains which do not carry a group a allotype or light chains of the lambda type. When fusions were carried out using a murine myeloma cell line (SP2/O-Ag14) which does not produce a murine immunoglobulin, only light chain secreting hybrids were observed. On the other hand, when a murine myeloma cell line (P3 X 63-Ag8 U1) which produces a murine L chain was used for fusion, 27% of the hybridomas resulting from these fusions secreted rabbit H chains. It was shown in at least one case that the rabbit H chain secreted from the fusion product is paired with a mouse L chain.

Although stability is a problem in the interspecies hybrids, several of the hybridomas produced in our studies have maintained ability to secrete rabbit Ig chains in culture and in the ascites fluid of athymic (nude) mice for periods greater than one year. Cytogenetic analyses were carried out on certain of the stable hybrid cell lines and no rabbit chromosomes were detected, indicating that translocation of rabbit genetic material to mouse chromosomes has occurred in the stabilization process.

All of the hybrids for which detailed allotype analyses could be performed expressed only allotypes present in the donor rabbit genotype. However, in two cases analysis by RIA revealed significant amounts of an unexpected or latent allotype which was synthesized along with a nominal or expected allotype. Unfortunately these clones stopped secreting before more detailed analyses could be conducted. These data indicated that extremely sensitive experiments designed to monitor such occurrences early after fusion could give insight into mechanisms governing latent allotype expression. Furthermore, the possibility of manipulating the spleen cell cultures prior to or during the fusion process can provide the opportunity to observe greater frequencies of nonexpected allotype expression.

We have isolated poly-A containing RNA from a series of rabbit-mouse hybridomas which secrete either a rabbit H or L chain. These mRNA fractions are from b4 and b5 light chain producers, a3 heavy chain producers, and an allotype negative, rabbit Ig^+ producer. Translations of these purified mRNA fractions are being carried out in both wheat germ and rabbit reticulocyte lysate systems, and the products of translation are being screened with rabbit anti-allotype antiserum, followed by SDS polyacrylamide gel electrophoresis and fluorography.

In order to be able to screen the cloned cDNA copies of our mRNA fractions, we have isolated pBR322 DNA containing the mouse kappa constant region sequence which was constructed in the Laboratory of Dr. Philip Leder (NICHD). In preliminary experiments this probe has been successfully nick-translated and is being used in a Northern blot analysis of rabbit DNA.

We have prepared high molecular weight DNA from the differentiated tissue (liver, spleen and brain) of rabbits of various allotypes, and from rabbit-mouse hybridomas which secrete a rabbit immunoglobulin chain, although no rabbit chromosomes can be detected in these cells. Using an artificial vagina we have collected ejaculates from both inbred and outbred rabbits of known allotype and pedigree. The sperm isolated from these ejaculates has been purified from most of the contaminating epithelial cells, and has provided an ideal source of high molecular weight genomic DNA.

Appendix

Contract N01 AI 02652 Maintenance of a Rabbit Breeding and Holding Facility.

This contract has objectives and use methods related to and in support of the above research project. In addition support is provided for LIG projects Z01 AI 00166-03 Genetic Studies on Rabbit Immunoglobulins and other Serum Proteins and Z01 AI 00166-03 Chemical Characterization of Rabbit Histocompatibility Antigens. Recently the contract has included rabbit holding, injection and bleeding for about 75 rabbits belonging to other (non-LIG) NIAID intramural scientists. The contract was held for 3 years by Borriston Research Labs and was transferred in July 1980 to Spring Valley Farms after competitive renewal.

Publications

Yarmush, M.L., Krutzsch, H., and Kindt, T.J.: Amino acid sequence analysis of immunoglobulin light chains by gas chromatographic - mass spectrometric techniques: structural identity of latent b9 and nominal b9 molecules. Molecular Immunol. 17: 319-326, 1979.

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Kindt, T.J., and Yarmush, M.L.: The significance of latent allotypes to regulation of immunoglobulin and histocompatibility gene complexes. In Atassi, M.Z. (Ed.): Critical Reviews in Immunology, CRC Press, Inc., in press, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00180-02 LIG

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Monoclonal Antibodies Directed Against Cell Surface Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. John A. Sogn	Chemist	LIG	NIAID
Others:	Dr. Thomas J. Kindt	Chief, LIG	LIG	NIAID
	Dr. Thomas Folks	Staff Fellow	LIG	NIAID

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1.0	0.8	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Monoclonal antibodies have been obtained in the past year by hybridoma techniques against the rabbit T cell tumor line RL-5 and against mouse histocompatibility antigen H-2.31. Antibodies to H-2.31 are being propagated to determine whether they can be usefully employed in structural studies.

The range of antigens under investigation has been broadened to include simple proteins, such as ovalbumin and insulin. Monoclonal antibodies to these proteins have been prepared for idiotypy studies.

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Monoclonal Antibodies Directed Against Cell Surface Proteins

Studies on cell line RL-5. Initial hybridoma experiments had as their object production of hybridomas against the rabbit T-cell line RL-5. This cell line is one of the few extant rabbit lymphoid cell lines. Lysates of radiolabeled RL-5 cells were used as the source of material for characterization and sequence of rabbit histocompatibility (RL-A) molecules in this Laboratory. The cell line was originated by Dr. M.D. Daniels by *in vivo* transformation of lymph node cells in an inbred rabbit with *herpesvirus ateles*. The cell line was not tumorogenic in the inbred rabbits or in nude mice, nor does it grow in soft agar. It carries the viral genome but does not shed virus. RL-5 does not have an Fc receptor or a C3 receptor. It is morphologically a medium lymphocyte and it is unresponsive to all mitogens tested. It reacts strongly with heterologous anti-rabbit T cell sera.

For a variety of technical reasons, RL-5 has proven to be difficult to raise hybridomas against and, for this reason, it did not serve very well as a proving ground for perfecting our hybridoma technique. However, a set of 5 hybridomas have been made and the specificity of these antibodies is now being tested. All of them bind to PBL's from the inbred donor strains, so none appears to have specificity for the transforming virus. None has been able to precipitate a molecule from a radiolabeled RL-5 cell lysate, although immunofluorescence assays localized reactivity to the cell membrane. Tissue distribution studies are in progress, as are further immunoprecipitation studies using limited fractions isolated from RL-5 lysates.

A very useful hybridoma would be one directed against an antigenic specificity of a rabbit histocompatibility (RL-A) molecule. Sequence studies using material immunoprecipitated with anti-$\beta_2\mu$ yielded only a single sequence. Presumably, the rabbit possesses 3 loci comparable to HL-A A, B and C or H2-K, D and L; it would be desirable to demonstrate this directly. So far, there is no indication that any of the mouse-mouse hybridomas made have the desired specificity. Because several of the rat anti-rat MHC monoclonal antibodies of Dr. Thomas McKearn appear to react with class I molecules of several species, collaborative experiments have examined whether these antibodies recognize class I molecules on RL-5. Although binding activity can be demonstrated against RL-5, all hybridomas tested (all of which are IgM antibodies) have failed to precipitate any protein from an RL-5 lysate, probably because of low affinity.

Other hybridomas. The production of hybridomas is valuable in experiments on idiotypy because it permits one to examine a single idiotypic specificity rather than a pool of idiotypes of indeterminate heterogeneity. It is hoped that monoclonal antibodies against ovalbumin and pork insulin prepared for experiments in collaboration with Dr. Thomas Folks will provide the tools necessary for the analysis of the antigen specific T cell response. Preparation of these antibodies proved to be valuable because it gave rise to a number of changes in the way we do our fusions and handle the positive clones. With these antibodies of predetermined specificity, the ELISA assay was inexpensive, quick and unambiguous, which is not always the case for antibodies to a cell line.

Hybridoma antibodies of high affinity are valuable and, in some cases, indispensable tools in the radiosequence analyses of mouse histocompatibility antigens in the Membrane Antigen Structure Section. For a first attempt to product such a needed antibody, the particularly difficult case of H2.31 (private specificity of $H2-K^d$) was chosen. Because of the d haplotype specificity, use of a BALB/c myeloma parent yielded a set of hybridomas with anti-self antibody activity. In preliminary studies, it appears that this anti-self activity is frequently associated with acceptable but abnormally low viability in culture and an inability to be propagated _in vivo_. Considerations of this amount of antibody required for sequence studies suggest that these will not be barriers to the production of adequate amounts of antibody, in that approximately 1-2 liters of culture fluid will suffice for sequence studies.

Appendix

Contract N01 AI 92612 - Establishment of a Tissue Culture Laboratory and Animal Facility.

This contract operates in support of this project and projects Z01-AI-00169-03, Primary Structural Analysis of Murine Transplantation Antigens and Z01-AI-00173-03, Nonallelic Expression of Genes Encoding Rabbit Immunoglobulins. The contract is located at Biotech Research Labs and has provided facilities for preparation maintenance and propagation of hybridoma cell lines.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00191-02 LIG

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Immunoregulation of T Lymphocytes - The Role of Anti-idiotype and Immune Complexes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. Kenneth W. Sell	Head, Immunobiology Section	LIG	NIAID
Others:	Dr. Thomas M. Folks	Staff Fellow	LIG	NIAID
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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒X(c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The injection of antigen into an animal results in the production of antibodies which specifically react with the immunizing antigen. Contained within the specificity of these antibodies are new antigens called idiotypes. Anti-idiotypic antibodies can be raised by immunizing animals with the original antibodies (idiotypes). An anti-idiotypic antisera was used to demonstrate crossreactivity between surface T cell receptors for soluble antigens (OVA) and a hybridoma anti-OVA antibody. Two other hybridoma anti-ovalbumin antibodies are being used as immunogens in goats in order to product additional anti-idiotypic sera. Also, splenic T cells from mice primed with blast T cells with anti-ovalbumin specificity are responsive in culture to mouse serum anti-ovalbumin. Other findings with rosette assays indicate that both B and T cell subpopulations from mouse spleens can bind ovalbumin, as well as can the hybridoma cell line. Anti-Ig binding rosettes are not observed with hybridoma cells although the secreted product is antibody and forms reverse plaques. Proliferation assays show a loss in responsiveness to specific antigens when unfractionated lymph node cells are tested, although fractionated "T" lymph node cells retain their proliferative function.

Immunoregulation of T Lymphocytes - The Role of Anti-idiotype and Immune Complexes

We will study B cell control of B and T cell activity by investigating idiotype-anti-idiotype interactions with both antibody and T cell receptor molecules. Specific objectives include: (1) Comparison of anti-idiotypic antibodies directed at T cell receptors and Ig receptors with similar specificity for soluble antigens. (2) Determine the kinetics of the appearance of autologous anti-idiotypic antibodies during the course of immunization and correlation of this with the time scale for formation of plaque forming cells and T cells that proliferate in presence of antigen.

Balb/C mice are immunized with ovalbumin in complete Freund's adjuvant and either spleen or LN cells are removed at varying times. T cells (nylon wool separated) are tested for proliferative capacity using either ovalbumin or a goat anti-mouse anti-ovalbumin (prepared by injection of hybridoma anti-ovalbumin). Splenic lymphocytes are used for fusion with the mouse plasmacytoma cell line (P3X63 Ag8U_1).

Direct, indirect, and reverse PFC from hybridomas and lymph nodes are detected by the Cunningham method. Rosette and plaque assays are carried out with SRBC tagged with the appropriate antigen (OVA-SRBC or goat anti-MIg-SRBC).

An IgE mouse hybridoma antibody was purified by affinity chromatography over an OVA-sepharose column and subsequently injected with complete Freund's Adjuvant into goats. Sera from these goats show precipitin lines against the immunizing antibody only and not against other mouse Ig.

This anti-idiotypic antibody stimulates ova primed splenic T cells *in vitro*, while no stimulation (blastogenesis) is observed from normal goat sera or against unprimed T cells. A proliferation index of 2-3X is seen following anti-idiotype restimulation. Following restimulation with antigen (OVA), a proliferation index between 5-6 fold is seen indicating some cross-specificity between humoral and cellular receptors. By utilizing an opposite approach, spleenic T cells from B/C mice, primed with T blast cells having ovalbumin specificity, can be restimulated in culture with affinity purified serum anti-ovalbumin antibody (200 g/ml). Findings from rosetting experiments indicate that 0.1 to 0.2 % of OVA primed T cells are positive for ovalbumin. Limiting dilution experiments, currently underway, should determine the percentage of receptor positive T cells in a primed population. Because such a small percentage of cells actually bind antigen, the observed proliferation in primed cell cultures is most likely due to recruitment of unprimed cells. Rosette experiments with purified T blasts should elucidate this question.

Hybridoma cells secreting anti-OVA have also been investigated for rosetting properties and plaquing capabilities using OVA-SRBC and goat anti-mouse Ig-SRBC. Indirect plaque assays with OVA-SRBC and reverse plaque assays with goat anti-mouse Ig-SRBC were developed with a rabbit anti-mouse Ig reagent. These assays enable us to determine specific anti-OVA antibodies secreted and total Ig secreted from either hybridoma cells or lymph node cells. Approximately 70% of our anti-OVA secreting hybridoma cells resette OVA-SRBC. The same percentage of cells form indirect plaques with OVA-SRBC and reverse plaques with goat anti-mouse-SRBC. This indicates that the hybridoma cells secrete an antibody with anti-OVA specificity and the receptor portion of the antibody is expressed on the surface of the cell. Less than 5% of the cells rosette with goat anti-mouse Ig-SRBC. This possibly indicates that the Fab portion of the anti-OVA Ig is exposed and the Fc portion remains buried in the membrane.

Kinetic measurements of PFC and cell proliferation indicate an inverse relationship. PFC responses (both indirect and reverse) remain low (300/10^6LNC) until day 9, peak at days 11-12 (1300-2000/10^6 LNC) and decline by day 14-16 (300/10^6 LNC). Eight weeks after priming PFC (specific) responses are still observed (300/10^6 LNC). Proliferation of unfractionated lymph node cells, however, show a stimulation against OVA as early as 4 days and are unresponsive after 3 weeks. Lymph node T cells, separated from B cells and supplemented with adherent presenting cells, are observed to retain their proliferative capacity for weeks after unfractionated lymph node cells have declined. Spleen cells produce no PFC against OVA but T cells begin to proliferate by day 8 and remain primed to restimulation for months.

These results support a concept of B cell regulation of T cell blastogenesis. If the decline in lymph node cell proliferative responsiveness is due to the increase in B cell production of Ig then the regulatory product could be antigen-antibody complexes or anti-idiotype.

In order to better compare the humoral antibody receptor with the T cell receptor it would be advantageous to develop an anti-idiotypic antibody to the T cell receptor. The quantity of purified receptor places some limitations on this undertaking. We plan on developing a procedure for cloning cells bearing T cell receptors either by production of T-cell hybridomas or by direct cloning methods utilizing in either case purified T-cell blasts obtained by multiple priming with antigen.

Once we have purified idiotypic and anti-idiotypic reagents, we will be in a better position to evaluate the possible regulatory influences of idiotype-anti-idiotype complexes on T cell function.

HLA restricted enhancement of natural killer activity. In a collaborative effort with the Immuno-oncology Division at Georgetown University Medical School (Dr. J.W. Woody and R. Hartzman) and Duke University Medical School (D.B. Amos), a project using human interferon as a boosting agent for Natural Killer (NK) activity was carried out and completed during the Fall of 1979. The results of the work, presented in the PLT Conference at NIH in April indicated that human interferon (HIF) has an enhancing effect on cryopreserved human peripheral blood lymphocytes (HPBL) for NK function and that this enhancing effect appears to be observed in a high percentage of patients bearing the HL-AA2 or HL-ADr4 specificities. Within families it was found that some parental haplotypes had an abrogating effect of the HIF enhancement on individuals possessing the A2 or Dr4 specificities.

Publications

None

LABORATORY OF IMMUNOLOGY
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PHS-NIH
Summary Statement
Office of the Chief
Laboratory of Immunology
October 1, 1979 through September 30, 1980

Allelic Variable Region Genes Specify Anti-Phosphorylcholine Antibodies

Immunoglobulin (Ig) heavy chains consist of both variable and constant regions. The former are responsible for the antigen-binding activity of these chains and are determined by variable (V) region genes (Igh-V genes) acting together with genes for the so-called J and D regions. A convenient marker for the study of the genetics of these chains have been the unique antigenic determinants, or idiotypes, expressed on the V regions of antibodies of a given binding activity. It is known that the antibodies produced by BALB/c mice upon immunization with certain phosphorylcholine (PC) containing antigens are quite homogeneous and often expresses an idiotype also expressed on several BALB/c myeloma proteins capable of binding PC. This idiotype is referred to as the T15 Id. Recently, a myeloma protein (CBBPL3 or C3) obtained from mice which possess the C57BL/6 Igh complex which has been shown capable of binding PC. This immunoglobulin while structurally very homologous to T15 (∿ 96% in the Igh-V region) expresses distinctive idiotypes. The T15 idiotype is not found in the anti-PC antibody response of C57BL/6 mice while the C3 idiotype is found in the response of C57BL/6 mice but not BALB/c mice. Segregation analyses, studies of allotype congenic strains and of recombinant inbred lines suggest that the expression of the two idiotypes is determined by allelic genes. This, together with their great structural homology, suggests that the T15 and C3 idiotypes are expressed on Igh-V regions which are the product of allelic Igh-V genes. The identification of allelic alternatives of an Igh-V gene provides a system for study the distribution and evolution of a given set of Ig gene products (Lieberman, LI/NIAID; Rudikoff and Potter, Laboratory of Cell Biology, NCI).

Expression of Allotypic Markers in Pre-B Cells

The B lymphocyte lineage is now believed to involve a sequential differentiation from stem cell to mature antigen-reactive cells. The earliest member of this lineage which may be identified and studied is the so-called "pre-B cell". The pre-B cell is characterized by the expression of cytoplasmic immunoglobulin (Ig) but not membrane Ig. In mice and humans, most pre-B cells express H chains without L chains. In rabbits, however, a substantial subpopulation of pre-B cells contain cytoplasmic H and L chains. Since pre-B cells lack membrane receptors, they should be insensitive to antigen-specific stimuli. The frequency with which a given Ig is expressed in pre-B cells should, therefore, be indicative of the rate at which that Ig is produced from the stem cell pool. This provided an opportunity to determine whether imbalances in Ig allotype, which are routinely seen, reflect intrinsic differences in generation rate of different Igs or their differential responsive to environmental antigens. This can be determined by enumerating the frequency in pre-B cells of the expression of given

Igs. At the κ (kappa) chain (b) locus, b4 Ig is produced in excess of b5 and b9 and b5 is produced in excess of b9. In contrast to these differences in serum antibody, pre-B cells showed coordinate expression of each of the b locus allotypes. This was also true for the a locus allotypes, which encode Igh-V regions. Similarily in the bas mutation which leads to marked reduction in expression of κ (kappa) chains, the frequency of pre-B cells expressing κ (kappa) chains was substantially greater than the relative amount of serum κ (kappa). These results indicate that the bulk of the allotypic variations in expression of Ig observed are the result of antigen-specific perturbations of the cell growth rather than intrinsic differences in the rate at which the Ig genes are activated. (Abdi and Mage, LI, NIAID, Cooper, Univ. of Alabama).

A New Mouse Model of Severe Immunodeficiency

Mice with defined genetic defects that lead to immunodeficiencies have been of great importance in understanding the normal function of the immune system as well as in clarifying the pathophysiology of human immunodeficiency. Two particularly important mutations are nu and xid. The nu gene in its homozygous form leads to congenital athymia and consequent ablation of the mature T lymphocyte system. The xid gene, which is found on the X chromosome, in its homozygous or hemizygous state leads to the deletion of a subpopulation of B lymphocytes and to unresponsiveness to a class of thymus-independent (TI) antigens designated type 2 TI (TI-2) antigens. Recently, the nu gene has been introduced into CBA/N mice, which possess the xid gene, and into their normal counterparts, CBA/Ca mice, which have a + allele at the xid locus. The resulting CBA/N nu/nu mice express a profound immunological defect, more serious than that seen in the presence of either defective gene alone. In particular, lymphocytes from these mice fail to respond to certain stimulants to which mice expressing the nu or xid determined defects alone respond normally. Thus, lymphocytes from CBA/N.nu/nu mice are unresponsive to a series of type 1 TI (TI-1) antigens, and to a series of B lymphoycte mitogens including lipopolysaccharide and Nocardia water soluble mitogen. The serum immunoglobulin levels are markedly diminished, being 5% or less than that of normal or of CBA/Ca.nu/nu mice. This is true for each of the major Ig classes. They have diminished numbers of splenic B lymphocytes and these cells express very little membrane IgD. Finally, peripheral lymph nodes from CBA/N.nu/nu mice are entirely devoid of lymphocytes and contain mainly stromal cells. The examination of these animals suggest that T cells are critical to the development of the B lymphocytes normally present in CBA/N mice. Further, they provide evidence that two independent lineages of B cells exist and that the abnormalities determined by nu and xid effect these different lineages, (Paul and Mongini, LI, NIAID, J. Mond, USUHS; I. Scher, Naval Medical Research Institute; J. Cossman, LP, NCI; and C. Hansen, VR, DRS).

Regulation of IgG Subclass Expression by T Lymphocytes

Recent studies suggest the existence of specialized T lymphocytes which determine the class, allotype, or idiotype of antibody produced by stimulated B cells. Unfortunately, the study of these T cell effects has been rendered quite difficult because a second set of T cell effects, those due to the carrier specific T lymphocytes, are also involved in these responses. Laboratory of Immunology scientists have used immune responses to thymus-independent (TI) antigens in an effort to simplify the study of T lymphocytes which regulate the type of antibody produced. To do this, type 2 TI (TI-2) antigens have been used because these molecules lack intrinsic polyclonal activation potential and the animals immunized have been *nu/nu* mice which have few if any mature T lymphocytes. Thus, when C57BL/10.*nu/nu* mice were immunized with the TI-2 antigen trinitrophenyl (TNP)-Ficoll, both IgM IgG antibodies of each of the main subclasses were produced. The amount of anti-TNP antibody in each of these classes was quite different and the hierarchy of responsiveness was IgM > IgG3 > IgG1 > IgG2b > IgG2a. This order is precisely the same as the 5' to 3' order for the *Igh* chain constant region genes and raises the possibility that the likelihood of gene switching is related to the distance from the postulated switch sites which are located 5' to the *Igh-μ* gene.

When the anti-TNP response of C57BL/10.*nu/nu* mice to TNP-Ficoll is compared to that of normal C57BL/10 mice, no difference in the IgM, IgG3, or IgG1 antibodies are seen. However, the IgG2a response of the normal is markedly greater than that of the nude and the IgG2b response slightly greater. This difference in responsiveness is not due to an innate deficiency of B cells from nude mice nor to any suppressive influence in the nude environment. Rather, it appears to reflect that absence of T cells at the time of immunization. Thus, if as few as 1x10^6 normal T cells are injected into a C57BL/O.*nu/nu* mouse immediately prior to immunization with TNP-Ficoll, the recipient expresses a major increase in its IgG2a response with no changes in its IgM, IgG3, or IgG1 anti-TNP response. This system provides a powerful approach to determine the mechanism through which T lymphocytes regulate Ig class expression (Mongini, Stein and Paul, LI/NIAID).

Thymic Antigen-Presenting Cells Determine Collaborative Phenotype of Developing T Lymphocytes

The activation of thymus-dependent (T) lymphocytes is a complex process in which the cells recognize both antigen and major histocompatibility complex (MHC) I-region encoded molecules (Ia molecules) presented on the surface of specialized antigen-presenting cells, which appear to be a subset of macrophages. In normal mice, T lymphocytes appear capable of recognizing the allelic forms of Ia molecules expressed on syngeneic but not allogeneic antigen-presenting cells. This "restriction" to the recognition of "self" Ia molecules is not a genotypic restriction; rather, it appears to be determined by the environment in which T lymphocyte precursors develop and, most particularly, on the thymus in which the bulk of this development occurs. Thus, if bone marrow chimeras are prepared by transfusing (AxB)F_1 bone marrow cells into lethally irradiated A recipients (F_1→A), the T lymphocytes which are obtained after repopulation of the recipients lymphoid tissue can recognize strain A Ia molecules as self and can be stimulated by antigen presented on strain A but not strain B antigen-presenting cells. Thus, the developmental environment, most particularly the thymus which is strain A in type, dictates the collaborative potential of the T lymphocytes. Recently, studies in the Laboratory of Immunology have revealed the presence within the thymus of a population of antigen-presenting cells. These cells are of hematopoietic origin and in irradiation chimeras they are replaced by donor cells; this process is rather slow and substantial numbers of donor antigen-presenting cells are not found until two months after transplantation. This finding raised the possibility that the cells which determine the collaborative phenotype might be of hematopoietic origin, most likely antigen-presenting cells, rather than of epithelial origin as was previously believed. To test this radiation chimeras of the type $(AxB)F_1 \rightarrow A$ were allowed to repopulate their lymphoid tissues. Two months later, when donor antigen-presenting cells were present in the thymus, peripheral T lymphocytes were depleted by treatment with anti-lymphocyte serum. When these mice repopulated their peripheral T cell population by cells which had been newly processed in the thymus, it was found that these cells could recognize antigen presented by either strain A or strain B antigen-presenting cells. This implies that the differentiative microenvironment of the thymus at that time allowed development of cells which recognize either A or B as self and, thus that the restriction phenotype is determined by cells of hematopoietic and not epithelial origin. Further the results strongly suggests that Ia-bearing antigen-presenting cells are involved in this process (Longo and Schwartz, LI/NIAID).

Cloning and Extended Culture of Antigen-Reactive T Lymphocytes

One of the major problems confronting cellular immunologists is the enormous heterogeneity of the cell populations with which they must work. Even in populations of T lymphocytes from hyperimmunized donors, the frequency of T lymphocytes specific for the immunizing antigen is no greater than 1 per 10^3 cells. This has made direct investigation of the consequences of the interaction of antigen with T lymphocytes and the regulation of this process very difficult. Efforts have been made over the last several years to obtain long term lines of antigen-specific and antigen-reactive T lymphocytes. During the past year, Laboratory of Immunology scientists have developed procedures which now allow the routine cloning of T lymphocytes specific for virtually any antigen. This procedure involves the initial immunization of inbred or F_1 mice with antigen emulsified in complete Freund's adjuvant, and the subsequent culture of T lymphocytes from these mice for three days in the presence of antigen and irradiated spleen cells, as a source of antigen-presenting cells. After this initial culture, the cells are distributed in soft agar containing antigen. These cells form colonies in the agar at a low frequency, approximatly 1 per 10^5 plated cells. The colonies which develop may then be picked and cultured in microwells in the presence of antigen, irradiated spleen cells, and a source of growth factor obtained from concanavalin A stimulated spleen cells. The latter is referred to as T lymphocyte growth factor (TCGF). These cells can be propagated over an extended time period and may be cloned in soft agar with an efficiency as high as 90%. The cells express exquisite antigenic specificity and restricted in their ability to be activated by the nature of the allelic form of Ia antigens expressed on antigen-presenting cells. A series of studies are now in progress in order to examine the characteristics, activation requirements, and receptors of these cloned T lymphocytes (Sredni and Schwartz, LI/NIAID).

Inhibition of T Cell Activation by Antibodies Specific for the Stimulating Antigen

The activation of specific T lymphocytes to proliferate as a result of exposure to antigen is a complex process. It is now recognized the antigen must be taken up and presented by a specialized cell. This cell, which appears to be a member of the monocyte-macrophage lineage, expresses Ia molecules and has been referred to as an antigen-presenting cell (APC). The T lymphocytes which are stimulated by antigen associated with APCs appear to be specific both for the stimulating antigen and for the allelic variant of the Ia molecule expressed by the APC. Indeed, Laboratory of Immunology scientists provided some of the earliest evidence for a role for Ia molecules in this process by demonstrating that antibodies to Ia molecules would block T lymphocyte activation. In view of the ease and regularity with which this anti-Ia induced inhibition was obtained, it has been difficult to understand why high concentrations of antibody specific for the stimulatory antigens has no inhibitory effect on activation. Indeed, this observation has led to the suggestion that antigen was not expressed on the surface of the APC. Recent observations have suggested that the failure of antibody to block might result from the antigen being present on the membrane of the APC in very low denisty such that antibody would be very unlikely to cross-link antigen molecules. It was predicted that if this were the case, the introduction of specific antigenic determinants on the surface of the APC which are also expressed on the antigen should allow antibody to inhibit. A preliminary confirmation was obtained when it was found that APC which had been trinitrophenylated 2 days earlier could stimuate proliferative responses by T cells sensitized against TNP on peritoneal exudate cells even in the face of a large excess of anti-TNP antibody. However, if additional TNP groups were introduced onto the cell surface immediately before adding antibody, the cells used rendered sensitive to inhibition. More decisive confirmation has come from exposing APC to soluble antigens, allowing them to "age" and then trinitrophenylating the cell, which leads to addition of TNP groups to a variety of membrane molecules, including antigen. Anti-TNP antibody now acts as a powerful inhibitor of activation. However, if an antigen is used which lacks sites capable of being trinitrophenylated, anti-TNP antibody has no blocking activity. These results indicate that antigen must be expressed on the cell surface in order to lead to activation and that, if cross-linking can be induced, antibody can be a most efficient inhibitor of activation. (Shevach, LI, NIAID).

Monoclonal Anti-Ia Antibodies Block Some But Not All Antigen Stimulated T Cell Responses

The activation of antigen specific T lymphocytes can be readily blocked by the addition of antisera to Ia antigens to the culture medium. This appears to reflect the need for T cell "self" receptors to interact with Ia on APC or the requirement for Ia to interact with antigen on APC membranes. In either case, it is not certain whether the same portions of the Ia molecule participate in these recognition events for responses to all thymus-dependent antigens or whether distinctive portions of the same molecule are responsible for responses to different antigens. To study this problem, monoclonal antibodies to Ia antigens of strain 2 guinea pigs have been prepared. These reagents were shown to have major inhibitory activity for responses to certain antigens and for mixed lymphocyte responses. However responses to other antigens, which also appear depend upon the very same Ia molecules, were not inhibited. This suggests that distinctive areas of the Ia molecule play roles in responses to different antigens and in turn that the Ia molecule plays a more sophisticated role than simply acting as a target for a self-receptor. Additional monoclonal anti-Ia antibodies are being prepared to further explore this finding (Shevach, LI, NIAID).

Diminished Autologous Mixed Lymphocyte Reactivity of Autoimmune Mice

In the last several years, it has been clearly shown that purified T lymphocytes from mice or humans when mixed with autologous non-T cells will undergo a proliferative response. This autologous mixed lymphocyte response (AMLR) is now believed to be due to the presence of T lymphocytes capable of recognizing and responding to antigenic substances expressed on autologous lymphoid cells. Furthermore, it has been suggested that the AMLR may reflect a fundamental immunological regulatory mechanism. In this regard, it is striking that patients with systemic lupus erythematosus (SLE) display markedly depressed AMLR's. Laboratory of Immunology scientists have begun a detailed study of the AMLR in mice in an effort to gain a fuller understanding of its true physiological significance. They have shown that the AMLR is mediated by a responding T lymphocyte and an Ia-bearing stimulator cell which resembles in its properties the antigen-presenting cell involved in activation of antigen-specific T lymphocytes. Furthermore, through the use of bromodeoxyuridine and light to eliminate proliferating cells, they have demonstrated that the cell which mediates the AMLR is distinct from the cell which mediates allogeneic (conventional) mixed lymphocyte responses. Efforts are in progress to prepare clones of both human and mouse T lymphocytes which mediate the AMLR.

The AMLR has been shown to be markedly diminished in autoimmune strains of mice which exhibit a syndrome similar to SLE. In particular, in $(NZBxNZW)F_1$ mice, the AMLR is markedly diminished in older animals which have active isease while it is quite normal in their younger counterparts who are elatively free of disease. Cell mixing experiments indicate that the defect results from an abnormality or deficiency in the responding population. Moreover, those cells which do respond in the autoimmune mice differ from normal in their phenotype of Lyt antigens. These model studies promise to aid in the determination of the significance of the AMLR in the normal and deranged immune response (Glimcher and Green, LI/NIAID; Steinberg/Arthritis Branch, NIAMDD).

Immunity to Cellular Antigens Limits Virus Growth

Membrane maturing viruses have been reported to incorporate membrane proteins, derived from the cells in which they grow, into their envelope. Among the molecules which have been found in viruses such as vesicular stomatitis virus (VSV) are histocompatibility antigens. Laboratory of Immunology scientists have studied the possible significance of the presence of histocompatibility antigens in the viral envelope by determining the effect of immunity to the cells used for the production of virus on the capacity of that virus to infect a recipient. It was initially found that VSV injected intraperitoneally or intravenously failed to grow in normal mice. A permissive state was achieved if the recipients had been given one of certain transplantable tumors, such as P815, a DBA/2 mastocytoma, or ascitic fluid from mice bearing such a tumor. Thus, DBA/2 mice which bore P815 would support the growth of VSV grown in P815 cells or in L cells, derived from H-2^k donors, equally well. By contrast, C3H mice pretreated and immune to P815, supported the growth of L-VSV to a much greater extent than the growth of P815-VSV. Similarly, DBA/2 mice pretreated with an H-2^k tumor supported the growth of P815-VSV to a much greater extent than that of L-VSV. In both experiments, infection with mixtures of the well growing virus and the poorly growing virus led to good virus growth in the recipient. These results indicate that immunity to histocompatibility antigens can profoundly effect the growth of viruses prepared in donor cells expressing that histocompatibility type. In turn, this suggests that the high degree of polymorphism among the histocompatibility genes and the high frequency of T cells specific for histocompatibility antigens may stem from repeated exposure to viruses expressing such antigens in their envelope. (Hecht and Paul, LI/NIAID).

Administrative, Organization and Other Changes

The Laboratory of Immunology continues to play an important role in the training of young scientists. During the past year Drs. Donna Sieckmann and Kathryn Stein, who were staff fellows, Dr. Dan Longo, who was a research associate, and Drs. Satoshi Toyoshima and Bronislaw Tyran, who were visiting fellows completed research and training programs in the Laboratory. Dr. Teresa Basham received her Ph.D. in genetics from the George Washington University for research carried out in the Laboratory under the supervision of Dr. Myron Waxdal. Each of these scientists made substantial research contributions during their tenure in the Laboratory of Immunology.

Drs. Kenneth Bernstein and Lawrence Samelson were appointed to the Laboratory as research associates. Drs. Tom Malek, James Jakway, Kenji Adachi and Anthony DeFranco joined the Laboratory as guest workers and Drs. Maureen Howard, Andrea Pavirani, Josianne Wantyghem and Makoto Iwata were appointed as Visiting Fellows.

During this year, the Laboratory was reviewed by the NIAID Board of Scientific Counselors, supplemented by several ad hoc reviewers.

Honors, Awards, and Scientific Recognition

Laboratory scientists play important roles in the national and international scientific community. Many serve on editorial boards of important journals. Dr. Paul is a member of the editorial board of the Journal of Immunology; Drs. Schwartz and Shevach are associate editors of this journal. Drs. Green and Shevach are referee editors of the Proceedings of the Society for Experimental Biology and Medicine. Dr. Mage is chairman of the editorial board of Federation Proceedings and is a member of the editorial; board of the Journal of Immunologic Methods. In addition, Dr. Paul is an advisory editor of the Journal of Experimental Medicine, member of the editorial board of the Scandinavian Journal of Immunology, Immunological Reviews, CRC Critical Reviews in Immunology, Human Immunology and the Journal of Clinical Immunology. He is an associate editor of Immunological Communications. Dr. Green is an associate editor of Clinical Immunology and Immunopathology and Dr. Inman is an advisory editor of Molecular Immunology.

Dr. Paul was the recipient of Founders' Prize for 1979 of the Texas Instruments Foundation, became President of the American Society for Clinical Investigation, and was elected to the Association of American Physicians. He completed a term as chairman of the Program Committee of the American Association of Immunologists and was appointed co-chairman of this committee. He is the co-chairman of the NIAID Study Group on Immunology and the U. S. organizer of the U. S. - Japan Symposium in Immunology. He is a member of the Scientific Review Board of the Howard Hughes Medical Institute. the Scientific Board of Visitors of the Oklahoma Medical Research Foundation, the American Cancer Society Committee on Personnel for Research, the Arthritis Foundation Fellowships Sub-Committee, and the Committee on Promotions and Tenure, NIAID. Dr. Paul was Weiser Lecturer for 1980 at the University of Washington, and presented invited lectures at the INSERM Conference on B-Cell Differentiation and Idiotypes, Seillac, France, the Armand Hammer Conference on Lymphocyte Development, La Jolla, CA, the Cancer Biology Symposium of the Given Institute, Aspen, CO and the Sympoisum on Primary Immunodefeciencies, Royaumont, France. He served as co-chairman of a workshop at the Fourth International Congress of Immunology, Paris, France.

Dr. Mage was a panel member and rapporteur for the Molecular Biology Panel of the NIAID Study Group on Immunology, is Vice-President of the District of Columbia Chapter of Sigma Xi, and competed a term as chairman of the NIAID EEO Advisory Committee.

Dr. Green was appointed chairman of the Program Committee of the American Association of Immunologists, after having completed a term as co-chairman of this committee. He is a member of the organizing committee of the forthcoming conference on Leukemic Markers, to be held in Vienna, Austria.

Dr. Shevach gave a major lecture at the Seventh International Convocation on Immunology, Buffalo, NY, was an invited speaker at the International Conference of Macrophages, Vienna, Austria, and was a workshop co-chairman at the Fourth International Congress of Immunology, Paris, France. He delivered invited lectures at the Workshop on Immunology and the Eye, Reston, VA and the Brook Lodge Conference on Immunology of the Skin. He completed a term as a member of the Allergy and Immunology Study Section of the Division of Research Grants, NIH. He is a member of the American Association of Immunologists Program Committee and was a panel member of the NIAID Study Group on Immunology.

Ms. Lieberman was an invited speaker at the NIH Conference on Homogeneous Antibodies and was a workshop co-chairman at the Fourth International Congress of Immunology, Paris, France.

Dr. Waxdal was an invited speaker at the American Society for Biological Chemists minisymposium on lectins and a session chairman and invited speaker at the International Union of Immunological Societies symposium on cell surface Receiptors in Immune Mechanism at Jablonna, Poland.

Dr. Inman was an invited speaker at the International Conference on Chemical Synthesis and Sequencing of Peptides and Proteins, at NIH.

Dr. Schwartz was an invited speaker at the Distinguished Lecture Series Wayne State University Department of Biochemistry and minisymposium speaker at the Federation of Amercian Societies for Experimental Biology in Anaheim, CA and was a workshop co-chairman at the Fourth International Congress of Immunology, Paris, France.

In addition, laboratory members presented seminars and lectures at universities and research institutes both in the United States and abroad.

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U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01-AI-00030-12 LI

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)
Antigen Recognition and Activation of Immunocompetent Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator: William E. Paul, M.D., Chief, LI/NIAID

Other Investigators:

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LAB/BRANCH
Laboratory of Immunology

SECTION

INSTITUTE AND LOCATION
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9	7	2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The study of the immunobiology of B lymphocytes in this research program has been based on two complementary approaches: 1) the analysis of genetic markers of mouse immunoglobulins and the regulation of their production; and 2) the analysis of B cell heterogeneity, function and activation through the study of mice with genetically determined defects and through the use of immunogenetic techniques to study regulation of clonal expression.

New efforts have recently been initiated to prepare monoclonal antibodies to differentiation antigens of B lymphocyte subpopulations and to clone mitogen and antigen reactive B lymphocytes.

Objectives:

The thymus-independent (B) lymphocytes are one of the two major subsets of lymphocytes; their principal function is the production of antibodies. The regulation of their activation is critical to the mounting of protective immune responses against a wide variety of pathogenic agents. Although the chemistry of the principal products of B lymphocytes, immunoglobulins, has been studied in detail, the genes underlying the synthesis of these molecules and the allotypic markers determined by alternative allelic forms of these genes have been the subject of renewed interest in recent years. This has been due both to the introduction of molecular genetic approaches which allow direct structural examination of these genes and the development of the realization that membrane immunoglobulins function not only as receptors for antigens but also as sites for the regulation of lymphocyte activation. Similarly, the recognition that B lymphocyte-subpopulations have distinctive properties, markers, and activation requirements has important implications for our understanding of the control of the immune response. The utilization of genetically defective mice and of genetic markers provides powerful tools to study these problems.

The Laboratory of Immunology research program on B lymphocytes has focused on these new opportunities to study regulation of antibody production and lymphocyte heterogeneity through a dual approach. This has included an analysis of genetic markers of immunoglobulins, and of the regulation of production of allotypic forms of immunoglobulins. At the same time, the study of B lymphocyte heterogeneity and activation has taken advantage of genetically defined systems such as those provided by mice with the x-linked immune deficiency (xid) gene and by the delineation of non-Ig, non-MHC genes which regulate clonal expression among B lymphocytes.

Methods Employed:

The techniques employed in the study of these issues include virtually all the current techniques of cellular immunology and of immunogenetics. Among the newer techniques which have been very important in our work are rapid flow microfluorometry and cell sorting using the fluorescence activated cell sorter (FACS) in the study of membrane immunoglobulins and, particularly, in the analysis of allotypic forms of IgD and of recombination within the Igh-C gene complex. Similarly, plate-binding radioimmunoassays, preparation of tritiated antibodies of high specific activity and production of monoclonal antibodies by hybridoma techniques have been employed. Techniques involved in efforts to clone B lymphocytes will be discussed in Results section.

Results:

A. Allotypic and Idiotypic Markers of Immunoglobulins and Analysis of their Regulation

1. IgD Allotypes

Immunoglobulin D (IgD) is now recognized to be a principal membrane (m) Ig molecule and, since it is formed in serum in very low concentrations, its main function appears to be that of a receptor molecule. LI scientists were the first to point out that IgD was found on a high proportion of human B lymphocytes and have actively studied the biology and chemistry of mouse and human IgD. A major effort has recently been made to examine the allotypic forms of IgD expressed by mice of each of the major Igh-C haplotypes. Using seven allotypic anti IgD determinants, rabbit anti-mouse δ^a, mouse anti-mouse δ^a, monoclonal antibody 10-4.22, monoclonal antibody 11-6.3, monoclonal antibody H6/31HLK, mouse anti-mouse δ^b, and mouse anti-mouse δ^e, we have classified the IgD of each strain in one of three types, IgD^a, IgD^b, and IgD^e. IgD^a is found in mice of the a,c,d,f,g,h,j, and n Igh-C types; IgD^b is expressed by mice of the Igh-C^b haplotype. IgD^e is found in mice of the Igh-C e and o types. Those studies have led to the recognition of two new Igh-C haplotypes, n and o. The n type encompasses the NZB and NZW strains, has also been described by the Herzenberg group, and represents a subdivision of the Igh-C^e haplotype, to which these strains were previously assigned and of which A mice are members. Igh-C^e mice have the e form of IgD while Igh-C^n mice have the a form. The o Igh-C haplotype is borne by mice of the AL/N and C.AL20 strains which were formerly classed, with AKR mice, as possessing the Igh-C^d haplotype. However, the d and o Igh-C haplotypes differ in that the Igh-C^d haplotype contains the gene for the a form of IgD while the Igh-C^o haplotype contains the e form of IgD. Since mice of Igh-C^o type express many idiotypic markers similar to those of Igh-C^e mice, these results suggest that the Igh-C^o haplotype arose by recombination between the d and e types and that the gene for IgD (Igh-Cδ) is closer to the Igh-V genes than are the genes for IgG (Igh-Cγ). The studies of IgD allotypes have also led to the definition of new IgD allotypic determinants, IgD.45 and IgD.46 and the localization of IgD.43 and IgD.44 on Fab fragments of IgD while IgD.36 and IgD.37 have been shown to be expressed on Fc fragments.

2. Regulation of IgG$_{2a}$ Expression

In our study of regulation of lymphocyte activation through allotypic and idiotypic determinants of membrane Ig, we have focused on two systems--the allotypic determinants of IgG$_{2a}$ molecules and the cross-reactive idiotype (IdX) expressed by the BALB/c TNP-binding myeloma protein MOPC 460.

The C57BL/6 (b) form of IgG$_{2a}$ has been shown by Herzenberg and her colleagues to be particularly susceptible to regulation by a T lymphocyte dependent mechanism which directly or indirectly distinguishes the b allelic form of IgG$_{2a}$ from the a allelic form of IgG$_{2a}$ and from the b allelic forms of other immunoglobulins such as IgG$_{2b}$. As an initial approach to characterizing the systems which might regulate production of specific classes and subclasses of Ig, we decided to examine antibodies to IgG$_{2a}^{b}$ for the existence of cross-reactive idiotypes. Our hope was that, if such cross-reactive idiotypes existed, they might also be represented on recognition sites of T lymphocytes which regulated expression of IgG$_{2a}^{b}$.

BALB/c, P/J, C.AL20 and DBA/2 mice immunized with the IgG$_{2a}^{b}$ myeloma protein CBPC-101 produce antiallotype antibodies which react with the IgG$_{2a}^{b}$ found in C57BL/6 serum. Anti-allotype antibody, purified from the serum of these mice and then conjugated to hemocyanin, was used to raise anti-idiotype antibody through the immunization of BAB.14, CXBI, or B.C8 mice. Anti-idiotype produced in each of these situations recognized an idiotypic determinant or set of idiotypic determinants shared by anti-allotype antibodies produced by a wide range of strains capable of responding to IgG$_{2a}^{b}$ allotypic determinants. This sharing of idiotypes suggests that the genes coding for V regions of anti-IgG$_{2a}^{b}$ antibodies are highly conserved. In addition, isoelectric focusing analysis indicates that the anti-allotype antibodies produced show limited heterogeneity and that the IEF patterns of the anti-allotype antibodies produced by all responder strains are very similar. The cross-reactive idiotypes (IdX) are also expressed on monoclonal anti-IgG$_{2a}^{b}$ antibodies kindly supplied to us by Oi and Herzenberg and by Bosma. Most interestingly, the IdX is expressed on monoclonal antibodies specific for *distinct* antigenic determinants on IgG$_{2a}^{b}$. Finally, although the capacity to make IdX^{+} anti IgG$_{2a}^{b}$ antibodies is found in mice of all *Igh-C* types tested, with the exception of *Igh-C*^{b}, it is controlled by an MHC encoded immune response (*Ir*) gene. Mice of the b, d, and s MHC types possess responder alleles of the *Ir-IgG*$_{2a}^{b}$ gene while mice of the k and a MHC types possess non-responder alleles of this gene. In parallel with these studies of the IdX's of anti-IgG$_{2a}^{b}$ antibodies, we are also undertaking similar studies of idiotypes of anti-IgG$_{2a}^{a}$ antibodies.

Functional systems are being developed to study regulation of IgG$_{2a}$ expression and eventually, to study presence of IdX on regulatory cells. Thus far, we have shown that C57BL mice immunized with the IgG$_{2a}^{a}$ myeloma protein UPC-10 develop suppressor T lymphocytes which eliminate the IgG$_{2a}$ response of B.C8 (C57BL.*Igh*^{a}) B lymphocytes primed to dinitrophenyl (DNP) antigens. This has been demonstrated by the transfer of T cells from UPC-10 primed C57BL mice together with carrier primed C57BL T cells and hapten primed B.C8 B cells to irradiated C57BL recipients. The suppression of IgG$_{2a}$ is specific; no effect on IgG$_{1}$, IgG$_{2b}$ or IgM antibodies, as measured by a plate-binding radioimmunoassay, is noted. A second regulatory system, which has particular promise involves the control of IgG$_{2a}^{b}$ anti-TNP responses to TNP-Ficoll and TNP-Levan by C57BL *nu/nu* mice. C57BL *nu/nu* mice make responses to TNP-Ficoll and TNP-levan which, when compared to responses of C57BL *+/nu* mice are deficient in IgG$_{2a}$ antibody but show enhanced amounts of IgG$_{1}$ antibody. T lymphocytes from normal C57BL mice transferred to *nu/nu* mice one day prior to immunization substantially increase the IgG$_{2a}$ anti-TNP response but have no effect on the IgG$_{1}$ or IgM anti-TNP responses. T lymphocytes from the allotype congenic strain, B.C8, not only fail to augment the IgG$_{2a}$ response of C57BL *nu/nu* mice but also inhibit the capacity of C57BL T cells to do so. This strongly suggests that normal B.C8 mice contain suppressor T cells which regulate expression of IgG$_{2a}^{b}$ anti-TNP antibodies. Such T cells will be useful ones upon which to test the effects of the anti-IdX antibodies specific for anti-IgG$_{2a}^{b}$ antibodies. Such studies will be carried out either in this system or in a comparable *in vitro* system if we are successful in establishing it.

3. Regulation of Expression of Id-460

A second general regulatory system which we have investigated is the expression of the idiotypic determinant of MOPC-460 myeloma protein in anti-TNP antibodies. MOPC-460 is a TNP-binding myeloma protein which expresses an idiotype designated Id-460. BALB/c mice immunized with TNP-levan, TNP-Nocardia water soluble mitogen (NWSM) or TNP-dextran make an anti-TNP response in which 10 to 20% of the antibody producing cells secrete 460-Id$^+$ anti-TNP antibodies. A similar result is obtained when BALB/c spleen cells are cultured with TNP-levan or TNP-NWSM. However, if T lymphocytes are removed from the spleen cell population, the fraction of anti-TNP plaque forming cells (PFCs) which produce Id-460$^+$ anti-TNP antibodies rises to 50% or more. The appearance of the 460-Id$^+$ PFC is suppressed by adding T cells from BALB/c mice, but not from allotype congenic C.B20 (BALB/c.$\underline{Igh}^b$) mice or C.AL20 (BALB/c.$\underline{Igh}^d$) mice. These suppressor cells can be removed from the T cell population by adherence to dishes bearing the MOPC-460 myeloma protein and cells recovered from these plates possess suppressor activity. The suppressor cell appears to possess the Lyt2 antigen and the Qa1 antigen. The expression of Lyt1 is not completely resolved.

These naturally occurring 460-Id specific suppressor T cells can be eliminated by acutely treating BALB/c mice with antibodies specific for idiotypes of anti-460 Id antibodies (so-called Ab_3) or by immunizing BALB/c mice with anti-460 Id antibodies. The elimination of 460-Id specific suppressor cells under these conditions is indicated by the finding that such mice, when immunized with TNP-levan or TNP-NWSM, express a substantially greater amount of 460-Id than do control mice. More decisively, T lymphocytes from such treated mice lack the ability to suppress the *in vitro* 460-Id$^+$ anti-TNP response by normal B cells or by B cells from "Ab_3" mice. This system is one of the few examples of natural regulation of an antibody response by anti-idiotype specific T cells and one in which the idiotype of the regulatory T cells is similar to the idiotypes of anti-460 Id antibodies.

These studies are being extended in several ways. Firstly, an alternative assay for 460-Id is being developed. We have established a plate binding assay based on the culture of 460 producing cells, such as MOPC460 tumor cells, in a TNP-coated microtiter well. Under such conditions, we can routinely detect the amount of protein produced by 30 myeloma cells and often that produced by fewer than 10 cells. In addition, we have recently obtained further evidence that the 460,anti-460,anti-(anti-460) antibody network is "normal" in that immunization of BALB/c mice with MOPC460 myeloma protein occasionally leads to the appearance of antibodies to the idiotypic determinants of anti-460 antibodies.

A major goal is to isolate the 460-Id specific suppressor in order to study its mode of regulation, its surface markers, its fine specificity, its histocompatibility restrictions and the nature of its receptors. One approach now underway is an attempt to clone 460-specific T lymphocytes from spleens of BALB/c mice bearing the MOPC-460 tumor and from BALB/c mice intentionally immunized with MOPC460 myeloma protein. To accomplish this, we are employing the soft agar T lymphocyte cloning procedure introduced by Sredni *et al*.

4. Effects of Anti-Immunoglobulins on B Lymphocytes

A direct test of the role of membrane Ig in regulation of B lymphocyte activation can be approached through the examination of the effect antibodies to membrane Ig on B lymphocyte responses. We have studied extensively the activation of B lymphocytes by antibodies to IgM.

Anti-μ antibodies are strong stimulants of B lymphocyte proliferation. This response is independent of T lymphocytes and, in the presence of 2 mercaptoethanol, requires few, if any, macrophages. Deaggregated anti-μ and $F(ab')_2$ fragments of anti-μ are equivalent to intact antibodies in their capacity to induce proliferative responses. Thus far, we have not been able to induce immunoglobulin synthesis with anti-μ, even in the presence of a variety of T lymphocyte supernatant factors including a concanavalin A supernatant, allogeneic effect factor or xenogenic effect factor. Indeed, the addition of T lymphocytes primed against the Fc region of goat immunoglobulin did not lead to immunoglobulin synthesis in response to goat anti-μ antibodies. Furthermore, anti-μ antibodies are powerful inhibitors of immunoglobulin synthesis induced by a variety of other agents. The dose response curves for stimulation of proliferation and for inhibition of Ig synthesis are virtually identical, suggesting that the two effects may be linked at least in certain cells.

Despite the fact that anti-μ can bind to virtually all B lymphocytes, it stimulates proliferative responses by only a subset of B lymphocytes. This was initially demonstrated by studies indicating that B lymphocytes from mice homozygous or hemizygous for the gene xid, which determines a functional B lymphocyte defect (see Section B), are unresponsive to anti-μ. Preliminary experiments suggest that treatment of B lymphocytes with anti-Lyb5.1 and complement eliminates the cells which respond to anti-μ. These experiments lead to the conclusion that the anti-μ responsive cells are members of the subset of B lymphocytes which bear the differentiation antigen Lyb5 and which are absent in mice with the xid determined immune defect.

B lymphocytes with differing degrees of responsiveness to anti-μ can also be isolated by separating cells on the Fluorescence Activated Cell Sorter [FACS] according to the amounts of IgM, IgD, and total Ig they express on their membranes. In general, B lymphocytes responsive to anti-μ bear relatively small amounts of IgM, intermediate to large amounts of IgD, and large amounts of Ig. Thus, the anti-μ responsive B lymphocyte has a high ratio of IgD to IgM. It is interesting in this regard, that the B lymphocyte defect caused by xid is associated with absence of proliferative responses to anti-μ and with the absence or diminished numbers of B lymphocytes which have a high ratio of IgD to IgM.

Our current studies of this phenomenon are centered around three areas. We have produced a series of monoclonal rat anti-μ antibodies through fusion of rat antibody producing cells with the BALB/c myeloma NS-1. We are now in the process of characterizing the specificity of these monoclonal antibodies and determining whether they are capable of stimulating proliferative responses. A second area of emphasis is that of producing cloned lines of B lymphocytes reactive to anti-μ (see Section C). Such cells should provide ideal materials for the study of the cell biology of B lymphocyte activation by anti-μ. It is the study of early events in B cell activation which constitutes the third major area in which study has recently begun.

B. B Lymphocyte Ontogeny, Heterogeneity and Activation

1. CBA/N Mice and the xid Gene

Our general approach to the study of the functional diversity of B lymphocytes has been based on the use of genetic systems. We have placed particular emphasis on the x-linked genetic defect of CBA/N mice which is characterized by a profound but discrete defect in B lymphocyte function. This defect involves the failure of CBA/N mice and mice with CBA/N x-chromosomes to respond to certain thymus-independent (TI) antigens. These include hapten derivatives of Ficoll, type III pneumococcal polysaccharide, and levan and dextran and their hapten conjugates. In contrast, mice with the CBA/N defect respond to another group of TI antigens, which includes the TNP derivatives of Brucella abortus (TNP-BA), lipopolysaccharide (LPS) and Nocardia water soluble mitogen (NWSM). On the basis of the distinctive responsiveness of CBA/N mice to these two sets of TI antigens, we proposed that they should be separately designated. TI antigens to which CBA/N respond are designated TI-1 antigens while those to which CBA/N mice do not respond are TI-2 antigens. In general, neonatal mice are unresponsive to TI-2 antigens but do respond to TI-1 antigens and alloanti-δ antibodies block in vitro responses to TI-2 but not TI-1 antigens. Finally, TI-1 antigens tend to be powerful polyclonal B lymphocyte activators whereas TI-2 antigens are, for the most part, inactive in this regard.

We have recently mapped the gene responsible for the CBA/N defect. This gene, termed x-linked immunodeficiency gene (xid), is found on the x-chromosome equidistant from the Ta (Tabby) and hyp (hypophosphatemia) genes.

2. The Lyb5 and Lyb7 Differentiation Antigens

Our working hypothesis is that the genetic defect of mice homozygous or hemizygous for the xid gene involves the deletion of a subpopulation of B lymphocytes. These deleted cells may either be mature members of a single B lymphocyte lineage or members of one of two distinct lineages of B lymphocytes. With this in mind, we produced antisera which identify the subpopulation(s) of B lymphocytes absent in mice with the xid determined defect. Although a variety of immunization procedures were tried, the one which was successful involved immunization of C57BL/6 mice with DBA/2 spleen cells. This serum, after absorption with DBA/2 thymocytes and spleen cells from defective (CBA/N x DBA/2)F_1 male mice, contains two types of antibodies, anti-Lyb5.1 and anti-Lyb7.1, which recognize antigenic determinants expressed on (DBA/2xCBA/N)F_1 male B lymphocytes but not on cells from the defective (CBA/NxDBA/2)F_1 male. The determinants recognized by these antibodies are each determined by an independently segregating gene. The Lyb5 gene is not linked to the H-2, Igh, Mls, or Lyb4 loci. The Lyb7 gene is also unlinked to the H-2, Mls, and Lyb4 loci, but it is loosely linked to the Igh gene complex being located approximately 20 to 25 centimorgans from the IgG_{2a} locus.

The Lyb5 antigen is detected by the ability of anti-Lyb5 antisera to lyse B lymphocytes in the presence of complement. By contrast, anti-Lyb7 antisera does not lyse B lymphocytes but it does inhibit the in vitro response of B ymphocytes to TI-2 but not TI-1 antigens. This result is particularly provocative because it indicates that a membrane molecule encoded by a gene linked to, but separate from, the Igh gene complex has an important role in regulating B lymphocyte activation.

We decided to search for monoclonal antibodies directed at markers of mouse B lymphocyte subpopulations, both for the intrinsic value of any such antibodies, and in the hope that we might find one which identified Lyb5 or Lyb7. To do so, we immunized Lewis rats with DBA/2 spleen cells or DBA/2 B lymphocytes, fused the rat cells to NS-1 and screened, using the FACS, for monoclonal antibodies that bound B cells, or subsets of them. Through this approach, we have obtained several anti-B cell antibodies, most of which bind all B cells. In some instances, these antibodies identify antigens expressed to different extents on B cell subsets, as indicated by the finding that cells from mice with the *xid* determined defect may express more or less antigen than control cells. Of these, three are of special interest. They are 17C9 which recognizes the ThB antigen; 14D10 which recognizes most Ig$^+$ cells and another group of Ig$^-$ cells which may include B cell precursors, and 14G8, which appears to be principally found on Lyb5$^-$ B cells. We are now in the process of a more precise determination of the cell types upon which these cells are expressed and in efforts to purify and to functionally test these cells.

3. Responses of xid$^+$ Mice to Polysaccharide Determinants

The study of the *xid* determined B lymphocyte defect of CBA/N mice has been a major interest of our laboratory for a considerable period of time. We have recently extended our study of this genetic model in several directions in an effort to gain greater insight into the nature of B cell development and function. The main areas we have investigated are the poor responses of mice with the *xid* defect to polysaccharide antigenic determinants, and the expression of more profound B lymphocyte defects by introduction of the *xid* gene into different genetic backgrounds.

It is long been known that mice with the *xid* defect are unresponsive to simple polysaccharides as immunogens and to hapten-polysaccharide conjugates. We have assumed that this failure to respond was due to the nature of polysaccharides as carriers not to an absence of B cells with receptors for polysaccharide determinants. Surprisingly, we noted that mice with the *xid* defect failed, under conventional immunization procedures, to respond to phosphocholine, as an antigenic determinant, on powerfully immunogenic carriers such as hemocyanin. This raised the possibility that either as part of their primary defect, or as a secondary effect, *xid*$^+$ mice might have very diminished numbers of precursors for antipolysaccharide producing cells. Indeed, Davie and his colleagues had proposed that the antipolysaccharide producing cells of normal mice might represent a subset of B lymphocytes because of their observation of a predominance of IgG$_3$ in the IgG response to a variety of polysaccharides and related antigens, such as α (1→3) dextran and phosphocholine. If a close relation existed between antipolysaccharide antibodies, IgG$_3$ and the *xid* defect, it would be anticipated that CBA/N mice would have little or no serum IgG$_3$. Accordingly, *xid*$^+$ and control mice were studied for their serum IgG$_3$ levels and for plaque-forming cells (PFC) secreting IgG$_3$. Such mice displayed amounts of serum IgG$_3$ which were approximately 10% of normal and an even greater depression in the number of IgG$_3$ PFC. This defect was observed in mice as old as 14 months of age. However, it was clear that *xid*$^+$ mice could produce some IgG$_3$. This was shown by immunizing them with 50 μg of lipopolysaccharide. They developed increases in both serum IgG_3 and in IgG_3 producing cells, although, in relation to normal controls treated in the same way, the levels were markedly depressed. This might indicate either that the IgG_3 anti-polysaccharide defect in xid^+ strains was secondary or that the xid^+ gene induced a defect which was only partial. In order to examine this point, we are attempting to determine whether the IgG_3 producing cells of mice with the xid determined defect are Lyb5 positive.

Another approach to the examination of the significance of the depressed antibody responses of CBA/N mice to polysaccharide determinants, even when presented on immunogenic carriers, has been a detailed study of responses to the polysaccharide α (1→6) dextran and its homologous hapten-hemocyanin conjugates, isomaltohexose-KLH (IM6-KLH) and isomaltotriose-KLH (IM3-KLH). CBA/N mice and $(CBA/NxDBA/2)F_1$ males, both of which display the xid determined genetic defect, fail to make antibodies upon immunization with dextran. When immunized with IM6-KLH or IM3-KLH, they make substantial primary responses. However, only a minority of the antibodies they produce bind dextran, although normal CBA mice immunized in this way make cross-reactive antibody. The antibodies the CBA/N's make do bind both IM3-BSA and IM6-BSA. These results indicate that the bulk of anti-dextran responses of normal mice to IM-6KLH should be a feature of $Lyb5^+$ B lymphocytes. Recent transfer studies have indeed shown that the capacity of B cells from $(CBA/NxDBA(2)F_1$ female cells to transfer responsiveness to irradiated recipients is ablated by pretreatment of these cells with anti-Lyb5 and C.

4. "Synergistic" B Cell Defects

The final two aspects of the work on xid determined unresponsiveness to be discussed in this report deal with the effect of introducing the xid gene into new genetic backgrounds. In the two examples to be discussed, mice are obtained which display more profound B lymphocyte defects than those normally associated with the xid gene. These animals should be of value both for the insight they may bring to the understanding of B lymphocyte development and for their value in providing model systems for evaluating the effect of other components of the immune system in "isolation".

The first of these models involves the introduction of the xid gene into a C3H background. Our initial observation was actually motivated by an interest in the possible synergy of xid with the Lps^d gene of C3H/HeJ mice. CBA/N females were crossed to C3H/HeJ males and the resulting F_1 females were mated to C3H/HeJ males. Approximately half of the male progeny of the back-cross, were unresponsive *in vitro* to all the B lymphocyte mitogens we tested (i.e. LPS, bacterial lipoprotein, NWSM) and failed to make anti-TNP antibody responses to either TI-1 or TI-2 antigens. These mice were more unresponsive than either of their parents; hence, we described their immune defect as a synergistic defect (SD). The SD phenotype required the presence of the xid gene, but not of the Lps gene. The SD phenotype appears to depend on the inheritance of one or more C3H genes, in a heterozygous form, together with xid in a homozygous or hemizygous form. SD mice have a diminished number of peripheral B lymphocytes and a very low mIgD to mIgM ratio. In the latter two respects, they are much more abnormal than other *xid*$^+$ mice. We are currently preparing inbred strains of SD mice by two strategies. In one, we are introducing the *xid* gene into a C3H/HeJ environment to make a congenic strain (C3H/HeJ.*xid*). We are now in the process of expanding strains following the ninth backcross generation in this breeding. In the second, we crossed an SD$^+$ backcross male mouse to a CBA/N female and have been intercrossing the offspring, testing them for the SD phenotype. Here, we are in the tenth intercross generation.

Our second model has involved the introduction of the *nu* gene into CBA/N mice. *Nu/nu* CBA/N mice of less than 12 weeks of age display an extremely profound immunodeficiency. Spleen cells from these mice are unresponsive to all mitogens tested thus far, including concanavalin A, phytohemagglutinin, LPS, bacterial lipoprotein and NWSM. They fail to respond *in vitro* to both TI-1 and TI-2 antigens and give very diminished *in vivo* responses to TNP-BA, the prototype TI-1 antigen. In most cases, 40% or fewer of their spleen are *mIg*$^+$ and by membrane labeling with ^{125}I and subsequent immunoprecipitation analysis, these cells express little, or no mIgD. Their peripheral lymph nodes are virtually devoid of lymphocytes, containing only stromal cells. The histology of their spleens is less well understood, but there are few if any typical lymphocytes. Finally, they exhibit a profound depression in serum concentrations of each class of Ig. These *nu/nu* CBA/N mice promise to yield major insights into the role of the thymus and thymus-dependent immunity in B cell development and will provide an exceptional model for the evaluation of "non-specific" immunity in protection to various pathogenic agents.

C. Other Genetic Models for the Study of B Lymphocyte Activation.

1. Regulation of Responses to Bacterial Levan

The immune response to bacterial levan (BL) and the myeloma proteins which bind this polysaccharide have been extensively studied by Lieberman and her colleagues. The response to BL consists of two sets of antibodies. One of these are specific for the principle fructosan linkage of BL, the β (2→6) fructosan; these are referred to as anti-BL antibodies. The second are specific for the branch point [β (2→1) fructosan] linkages, which are also found in inulin (In). These antibodies are referred to as anti-In antibodies. Most of the myeloma proteins which bind BL also bind In and express one or more of a family of cross-reactive idiotypes (IdX's) which are also found on the anti-In antibodies. The ability to produce anti-In antibodies in response to BL and to develop the IdX is dependent on genes in the *Igh-V* gene complex of BALB/c.

We have recently investigated the IgG anti-In antibodies produced by BL immunized BALB/c mice. IEF analysis indicates the presence, in the serum of essentially all BALB/c mice, of similar anti-In antibodies, which are of very limited heterogeneity. Their IEF pattern may be regarded as a single "spectrotype" consisting of three prominent bands and two minor bands. C57BL/6 mice make no detectable IgG anti-In antibodies after such immunization. However, F$_1$ hybrids between BALB/c and C57BL/6 make a response which is substantially greater in diversity upon IEF analysis. This response appears to reflect the action of a C57BL gene on the *Igh*^{a} determined response and not the development of an Igh^b determined response under the influence of a BALB/c gene. This is indicated by the finding that the C57BL.Igh^a congenic strain B.C8 makes anti-In antibodies with comparable diversity to those of F$_1$ mice while the BALB/c.Igh^b congenic strain C.B20 make no anti-In antibody to BL. When F$_1$ mice are backcrossed to BALB/c mice, approximately half the progeny make anti-In antibodies in response to BL which are more heterogeneous than those of BALB/c. This suggest that one C57BL gene is mainly responsible for in the increased heterogeneity of the Igh^a anti-In response to BL. This gene, designated spectrotype regulatory gene-1 (Sr-1), is not linked to the Igh gene complex, to the MHC or to the coat color genes that distinguish BALB/c and C57BL/6. Finally, it appears unlikely that Sr-1 codes for L chains since BALB/c mice immunized with In-BA or In-KLH make a diverse anti-In response. We are directly examining the effect of κ genes by using C57BL/6 mice congenic for the Ly3.1 marker, which is linked to Igk, in a backcross test. Our current interpretation is that Sr-1 is a member of a family of genes which, through still unknown mechanisms, determines which of the clones potentially responsive to BL is actually activated.

2. Regulation of Nonspecific B Cell Recruitment by Ir Genes

It is well known that the activation of most T lymphocytes requires that the T cell recognize both antigen and an MHC gene product displayed on the surface of a specialized cell, often referred to as an antigen-presenting cell (APC). The APC is believed to be a macrophage. The interaction of T lymphocytes with B lymphocytes which leads to the helper phenomenon also depends, in most cases, on the recognition of both antigen and an MHC gene product on B cells. Closely related to this "histocompatibility restriction" in the interaction of specific T lymphocytes with antigen-bearing APC and B cells is the realization that specific immune response (Ir) genes act in both APC and B cells. That is, that APC and B cells from non-responder individuals do not interact with antigen-specific T cells, even if the latter are capable of responding. We have recently encountered a paradoxical situation in which T lymphocytes display an Ir restricted capacity to non-specifically activate B lymphocyte proliferation.

We noted that the addition of small numbers of antigen-primed T lymphocytes to B lymphocytes from non-primed donors causes substantial B lymphocyte proliferation in the presence of antigen. Indeed, there is no greater proliferation if the B lymphocytes are derived from primed donors, strongly suggesting that B cells are recruited to proliferate independent of the specificity of their receptors. By contrast, the T lymphocytes must be obtained from an immunized donor or no response ensues. If one utilizes the response of mice to the polymer of glutamic acid, alanine, and tyrosine (GAT), the response to which is regulated by an Ir gene, a similar result is noted when B and T lymphocytes are drawn from the same donor. However, if one prepares an F$_1$ hybrid between responder and non-responder strains, such as a (B10xB10.S)F$_1$, primed T cells from the F$_1$ recruit B10 (responder) but not B10.S (non-responder) B lymphocytes to proliferate. This Ir restriction cannot be explained by postulating that the B lymphocytes are the source of the APCs, since the same result is obtained in the presence of added irradiated F$_1$ spleen cells, which are a rich source of APCs. Even more decisively, if one cultures F$_1$ T cells with a mixture of B10 and B10.S B lymphocytes, virtually all the proliferation is a feature of the B10 B cells. These results strongly suggest that Ir restriction in the activation of B lymphocytes may not require that T cells see Ir gene products and antigen-bound to B cell receptors. These data could be interpreted to reflect the ability of T cells to recognize Ir gene products (? Ia antigens) alone in certain physiologic stiuations. More likely, the B cells may have bound small amounts of GAT non-specifically, which may have been sufficient to allow their recognition by specific T cells. In either case, a mechanism through which Ir restricted non-specific collaboration might lead to substantial thymus-dependent activation of B lymphocytes is indicated by these results. Efforts to choose between the alternative explanations are now in progress.

D. Efforts to Clone Stimulant Reactive B Lymphocytes.

One of the major limiting features in the analysis of lymphocyte responses to any stimulant, be it an antigen or a mitogen, is that the response is a feature of only some of the cells in the population being studied. This has made direct biochemical evaluation of lymphocyte responses difficult and, to a large degree, is responsible for the paucity of information which we have about certain early phases in B lymphocyte responses and about the chemical basis of stimulant induced proliferation, differentiation, tolerance, and regulation. B lymphocyte tumors hold the promise of providing homogeneous cell types, but since they fail to respond to many stimulants, they yield an essentially static picture of these cells. Recent success, both in the Laboratory of Immunology and elsewhere, in cloning and propagating antigen-reactive T lymphocytes has raised the possibility that similar clones of B lymphocytes could be prepared. Thus far, we have prepared B lymphocytes both by positive and negative selection, cultured them in liquid media for two to three days with a variety of stimulants including LPS, anti-μ, anti-idiotype antibody, and antigen. The cells are generally placed in soft agar, together with the stimulants, and colonies picked when they appear large enough in size. The cells are then cultured in microtiter wells with one or more growth factors, including a concanavalin A supernatant. Several such lines are growing, some for a substantial length of time (more than 4 months). Our current goal is to determine whether they are actually B lymphocytes. A variety of approaches to this end are being used.

E. Role of Host Factors in Regulating Growth of Vesicular Stomatitis Virus (VSV).

It has previously been reported that membrane maturing viruses, such as VSV incorporate some host proteins, such as histocompatibility antigens, into their envelope. In an effort to test the immunologic significance of this observation, we have determined the capacity of VSV prepared in L cells, which are $H\text{-}2^k$ in type, or in P815 cells, which are $H\text{-}2^d$, to grow in $H\text{-}2^d$ mice or in $H\text{-}2^k$ mice. In particular, we wished to determine whether immunization against histocompatibility antigens of the cells in which the virus had been prepared would limit subsequent growth of that virus. We initially observed that inoculation of normal mice with VSV does not lead to the development of either viral plaque forming units (PFU's) or plaque forming centers (PFC's). However, if these mice bear tumors such as P815, or have received ascitic fluid from a tumor bearing mouse they are now subject to infection by VSV. Using such "permissive" mice it can be shown that immunization of $H\text{-}2^d$ mice with $H\text{-}2^k$ tumor cells will limit the growth of L cell ($H\text{-}2^k$) VSV compared to P815 ($H\text{-}2^d$) VSV. Similarly immunization of $H\text{-}2^k$ mice with $H\text{-}2^d$ tumor cells limits the growth of P815 ($H\text{-}2^d$) VSV compared to that of L ($H\text{-}2^k$) VSV. In each case, mixtures of the two types of virus grow comparably to the better growing virus. This indicates that an intimate relation exists between the virus and the cellular antigen against which immunity is directed. A similar result has been obtained in an in vitro test system. We conclude that immunity to alloantigens may offer a degree of protection against viruses grown in cells expressing those antigens and that the expression of such antigens on viruses may have had an important evolutionary role in determining the polymorphism of and reactivity to histocompatibility antigens.

In the course of carrying out these studies we also noted that treatment of VSV infected cells with a source of complement could lead to the lysis of these cells. This phenomenon does not require the presence of antibody, is mediated by the classical complement pathway, and can be achieved before virus particle assembly has been completed. It appears to reflect the fixation of C_1 by VSV G protein on the surface of an infected cell, by a mechanism akin to the fixation of C1 to G protein in the VSV envelope. This complement determined lysis of virus infected cells may provide an important non-antigen specific immunologic defense.

Significance to Biomedical Research

These studies, aimed as they are at uncovering the fundamental cellular and genetic mechanism underlying antibody production, promise to provide new insights into the ability to more precisely control immune responses. In turn, this should allow one to amplify responses that are normally too limited to provide protection against certain classes of pathogenic microorganisms and tumors and to limit responses in instances of autoimmunity. Both of these goals clearly would be of importance in control of disease states.

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Lymphocyte Interactions, Receptors and Functions

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SUMMARY OF WORK (200 words or less - underline keywords)

We are first investigating the autologous mixed lymphocyte reaction (AMLR). In strains of mice with auto-immune disease, and the nature of the stimulating cell and the antigen that is recognized in this reaction. We are also investigating the cellular immune defects in patients with systemic lupus erythematosus (SLE). Lymphocytes from these patients fail to demonstrate normal proliferative and cytotoxic responses to TNP-modified target cells. Lymphocytes from normal individuals treated with plasma from patients with SLE led to augmented proliferative and cytotoxic responses suggesting that such treatment eliminated suppressor T cells. We are also studying the mechanism of fibroblast activation in cell mediated disorders. Here allogeneic mixed lymphocyte reactions are being generated, supernatants from this reaction are capable of inducing fibroblast proliferation. This may be the in vitro counterpart of the scleroderma like syndrome observed in chronic graft versus host disease. A B cell leukemia (L$_2$C) of inbred strain 2 guinea pigs is being investigated as a model of human leukemia. The chemical nature of the tumor specific transplantation antigen investigated by immunization protection tests in syngeneic animals.

Project Description

The autologous mixed lymphocyte reaction (AMLR) is the *in vitro* proliferation that occurs when T lymphocytes are co-cultured with irradiated syngeneic non-T cells. This reaction has been shown to have immunological specificity and memory. In humans the AMLR has been shown to be absent in several diseases including the auto-immune disease lupus erythematosus (SLE). There is also evidence that the T lymphocyte that proliferates in the AMLR may act as a cell which suppresses the immune response. Since the AMLR may have important immunological significance a number of studies are being performed to further analyze this phenomenon.

Objectives:

A) Study of the AMLR in strains of mice that have autoimmune disease.

Methods Employed:

T cells obtained from lymph nodes were used as the responding cells and syngeneic irradiated peritoneal exudate cells were used as the stimulator cell population. The proliferation was measured by ^{3}H thymidine incorporation at 5-6 days.Three strains of mice with autoimmune disease were examined,

Major Findings:

The data obtained demonstrates that the AMLR is present in young mice of these three strains but is greatly reduced in their older counterparts. We have determined from reciprocal mixing experiments that the defect in the older mice resides in the responder cell population and is probably not the result of an active suppression of that population. We have also defined the phenotype of the responding cells and have identified some of the surface characteristics of the stimulating cells in the AMLR of (NZB x NZW)F_1 mice.

Objectives:

B) Examination of the specificity of the AMLR.

Methods Employed:

The autoreactive T cells will be cloned using techniques developed by B. Sredni. T cells are first stimulated in an AMLR by non T cells, isolated and cloned in soft agar and then grown up in liquid culture (with the help of T cell growth factors). These clones of T cells can then be tested for their ability to respond to syngeneic stimulating cells, allogeneic stimulating cells, as well as different protein antigens. The cells proliferating in an AMLR can also be selectively eliminated by treatment with BuDR and light and the function of the remaining cells can be examined.

In a related study the concept being examined is that the AMLR reflects an interaction of self with self and that this reaction requires previous learning in the thymus of the sort described by Zinkernagel and others. Thus one would predict that F_1 cells educated in a chimeric mouse bearing a thymus of one parent might no longer "recognize" in an AMLR, a stimulator cell belonging to the non-thymic parent. Preliminary experiments suggest that this is the case and should allow us to identify the nature of the stimulating antigen by using recombinant inbred strains.

Major Findings:

The T cell responding in the AMLR can be eliminated with BuDR and light treatment; this results in the elimination of the response to the foreign antigen PPD but not the response to allogeneic stimulator cells or mitogens.

Significance to Biomedical Reaearch:

The autologous mixed lymphocyte reaction may be a manifestation of an important immunoregulatory mechanism. The selective loss of the AMLR but not the allogeneic MLR in SLE, Hodgkins disease and primary biliary cirrhosis (in whom a loss of suppressor T cells is also observed) may be an important new clue as to the etiology of certain human autoimmune diseases. The loss of the AMLR in the mouse models of human autoimmune diseases is highly provocative and also allows a sophisticated examination of the nature of this defect in autoimmune disease.

Pathogenesis of Systemic Lupus Erythematosus

Objective:

Analysis of the Cellular Immune Defects in Patients with Systemic Lupus Erythematosus.

Methods Employed:

Peripheral lymphocytes and plasma from patients with SLE, as well as those of normal subjects are used. For separation of T cells, Ficoll-Hypaque gradient techniques are used after rosetting with sheep red blood cells. Fc-receptor bearing T cells (T·cells) are separated by rosetting with IgG antibody coated ox red blood cells. Trinitrophenyl sulfonic acid (TNPBS) is used to modify the surface of autologous cells.

After cultivation of T cells or non-T-cells with autologous TNP-modified cells, their proliferative and cytotoxic activitites are measured by ^{3}H-thymidine uptake and ^{51}Cr release from TNP-modified autologous cells, respectively. The ability of lymphocytes from patients with SLE to perform these functions were compared to lymphocytes from normal individuals.

In another study plasma from patients with SLE were tested for their ability to mediate antibody dependent cell mediated cytotoxicity (ADCC) against normal T cells. Effector and non-T-cells were obtained from normal individuals as well as from patients with SLE. The reason for doing this study was because patients with active systemic lupus erythematosus (SLE) often have circulating antibodies to T cells. These patients also often have leukopenia and diminished numbers of T lymphocytes. In addition, certain T lymphocyte functions are frequently impaired in patients with SLE.

It has been previously considered that a complement-dependent cytotoxic mechanism was responsible for the above observations. We wished to demonstrate that ADCC, a cytotoxic reaction mediated by antibody and effector cells in the absence of complement, could also kill normal T cells.

Major Findings:

Pure T cells obtained form the peripheral blood were examined for their proliferative and cytotoxic responses against trinitrophenyl (TNP)-modified autologous non-T cells. Primary and secondary proliferative T-cell responses to TNP-non-T cells were markedly impaired in patients with active systemic lupus erythematosus (SLE), as compared to normal subjects or patients with inactive SLR. On the other hand, normal T cells pretereated with active SLE plasma and complement showed significatnly greater primary and secondary proliferative responses against autologous TNP-non-T cells than those pretreated with autologous, normal allogeneic, or inactive SLE plasma. These augmenting effects were observed only with the IgM fraction of the plasma from active SLE patients.

The treatment of normal T cells with active SLE plasma led to the generation of secondary cytotoxic activities against TNP-modified autologous cells. These enhancing effects of active phase plasma on these proliferative and cytotoxic TNP responses were also seen, when the T cells from inactive SLE patients were pretreated with their own active phase plasma. The SLE plasma-induced augmentation of proliferative TNP responses of normal T cells were inhibited by adding normal T cells. The above results suggested that treatment of normal T cells with active SLE plasma-killed subpopulations of suppressor T cells leading to increased TNP responses.

With regard to the ability of plasma from patients with SLE to mediate ADCC we observed that only the plasma of those patients with active SLE, and in whom anti-T cell antibodies could be demonstrated by the more classical complement-dependent cytotoxicity, were able to mediate such an ADCC reaction. The most efficient effector cell population for this reaction was unfractionated mononuclear cells; T cells themselves were ineffective. Since a single SLE plasma could induce ADCC in T cells from several different unrelated individuals, it is unlikely that antibodies directed against particular HLA antigens or blood group antigens are involved.

Study of the plasma from several pateints obtained during the active and inactive phase of the disease demonstrated that ADCC could be mediated only from plasma obtained during the active phase of disease. The IgG fraction of the plasma was responsible for inducing ADCC and aggregated IgG could block the reaction. The fact that the IgG fraction was often more effective than the unfractionated plasma suggested that immune complexes present in SLE plasma might partially block the expression of ADCC.

Significance to Biomedical Research:

These observations suggest that the antibodies to T lymphocytes among the many auto antibodies found in patients with SLE, may play an important role in perpetuating the disease process by interfering with naturally occuring suppressor T cell functions, which interference may in turn lead to increased auto antibody production, thus perpetuating a disease producing cycle. These present studies have also demonstrated that antibodies to T cells may also cause a loss of T cells by a mechanism independent of complement action, namely antibody dependent cell mediated cytotoxicity. Such a loss of T cells may also lead to an immunological imbalance again leading to perpetuation of the disease process.

Our results would strongly suggest that methods to specifically remove antibodies reactive with T lymphocytes from the plasma of patients with SLE might have theraputic effects.

Studies of the Guinea Pig L_2C Leukemia and a New Myelogenous Leukemia:

The L_2C lymphatic leukemia of inbred strain 2 guinea pigs arose about 25 years ago in an old female strain 2 guinea pig. Studies in our laboratory starting 8 years ago have shown that this leukemia cell is of B cell origin and has surface IgM and C3 receptors. There are several variants of this leukemia; one variant exists which lacks Ia antigens (an antigen of the major histocompatibility complex of the guinea pig). Our laboratory has demonstrated that, as measured by immunization protection tests in inbred strain 2 guinea pigs, the L_2C cell has a powerful tumor specific transplantation antigen (TSTA). However, in the Ia negative variant of the L_2C cell, this TSTA is not immunogenic. Our previous studies suggested that the TSTA is not related to the surface IgM molecule.

The main goal of the present study is to identify the chemical nature of the TSTA of the L_2C leukemia cell. A 3M KCl extract of the L_2C cell contains the TSTA as measured by immunization protection tests in syngeneic strain 2 animals. Immunization with as little as 100 μg of this material per animal confers protection against a lethal challenge of L_2C cells. Fractionation of the KCl extract on Sephadex G-200, DEAE cellulose and CM cellulose indicate that the immunogenic molecule has a M.W. of less than 20,000 and is basic. The material is very resistant to heat and extremes of pH but is destroyed by treatment with trypsin, neuramnidase and periodate. These properties suggest that the TSTA is a low M.W. glycoprotein. Attempts to further fractionate the material by polyacrylamide gel electrophoresis were unsuccessful, none of the fractions eluted from these gels were immunogenic.

Most recent studies have used CM cellulose followed by G-50 chromatography. Several immunogenic regions from G-50 chromatography have been obtained and are now being currently investigated. In addition, Biogel chromatography is being performed on fractions obtained from CM cellulose to obtain better resolution of the immunogenic fractions. Progress in the chemical identification of the TSTA of the L_2C cell has been slow because each individual assay takes 7-8 weeks to complete (*in vivo* protection being the final end point).

Studies of the new myelogenous leukemia that originated and which grows in strain 13 guinea pigs have been difficult to continue because it appears that the biological behavior of this tumor has changed. The tumor now grows very slowly at the site of local intradermal or subcutaneous ection and often the tumor either regresses spontaneously or the animal does not develop systemic leukemia. Attempts to pass the leukemia by either the intraperitoneal or the intravenous route have not proved to be successful.

Significance to Biomedical Research and the Program of the Institute:

The properties of the TSTA of the L_2C leukemia cell are highly unusual and differ from the properties of most other TSTA described by other investigators. Most other TSTA have a M.W. greater than 40,000 daltons and are sensitive to heat.

The fact that this TSTA is on a malignant B lymphocyte is of particular interest; once the L_2C TSTA is more completely identified one could look for a similar TSTA in several different human B cell Leukemias. Another possibility is that this TSTA could represent a marker for a particular stage of B cell differentiation present on only a very small number of normal B cells.

Pathophysiology of Fibrosis in Immune Disorders:

We are interested in the mechanisms of fibroblast activation in cell mediated immune disorders. We have chosen the prominent dermal fibrosis observed in patients with chronic graft versus host disease as a prototype and have utilized the mixed lymphocyte reaction as an *in vitro* method of generating factors capable of stimulating fibroblast proliferation and product biosynthesis.

Specifically, we intend to characterize the factors produced by human mixed lymphocyte reactions which are capable of stimulating fibroblast proliferation and collagen biosynthesis. The cell of origin, kinetics of secretion, modes of modulation, and biochemical characterization are targeted goals of this project. We intend to clearly distinguish these factors from others produced by activated mononuclear cells, e.g. T-cell growth factor and fibroblast chemotactic factor [LDCF-F] on a functional and molecular level.

Other objectives include studying the effect of these factors on fibroblast proliferation, product biosynthesis, and migration. Also, we intend to determine if product (collagen) biosynthesis can be separately triggered independent of a stimulus to proliferate. Also, we plan to study the differential effect of these factors on papillary and reticular fibroblasts, two distinct subsets of dermal fibroblasts with different growth characteristics. Also we intend to determine the target specificity of the isolated factors, i.e., can the isolated factors influence the behavior of other cell types, e.g. endothelial cells, keratinocytes, and fibroblasts from other species. Furthermore, we intend to detect the presence of receptors on responding target cells and characterize factor-receptor interactions.

Methods Employed:

Multiple aliquots of frozen human dermal diploid fibroblasts from the same normal individual and of the same early passage number are obtained from the American Type Culture Collection. Endothelial cells are obtained by collogenase treatment of human umbilical vein and passaged serially. Fluorescent staining for Types I and III collagen and factor VIII, respectively, confirm the homogeneity and identity of these cultured cells.

Standard methods for primary and secondary MLR's are followed using PBM from normal unrelated volunteers. Target cells are grown in microtiter wells and proliferation is measured by using automated harvesting equipment and standard tritiated thymidine incorporation techniques. Total DNA per well is measured using a photometric diphenylamine assay. An ELISA assay is used to measure total soluble collagen and fibronectin synthesis per well of cells. Fibroblast migration is measured in a blindwell Boyden chamber using gelatin coated polycarbonate filters. Absorption experiments on formalinized fibroblasts, endothelial cells, and lymphoblasts are designed to study target specificity and factor binding characteristics.

Major Findings:

Secondary human allogeneic MLR's generate a factor that stimulates human dermal fibroblasts to proliferate in vitro. On gel filtration columns, this factor has an apparent M.W. of 120,000 daltons. Secondary MLR's are significantly more efficient than primary MLR's in producing this factor. This enhanced efficiency of production has been shown to be due to the presence of primed T lymphocytes. However, cell enrichment and depletion experiments suggest that the factor is ultimately a MΦ product, the production of which is enhanced by the addition of primed allogeneic T lymphocytes. Factor production occurs within 12 hours of initiating the MLR and is not inhibited by abrogation of lymphocyte proliferation with mitomycin C. Factor production is serum dependent and can be modulated by the addition of agents which block prostaglandin biosynthesis and also by the addition of serine protease inhibitors to the MLR culture system.

In the presence of factor fibroblasts continue to proliferate at an accelerated rate, while controls demonstrate contact inhibited growth. Excess fibroblast proliferation in response to factor is dose dependent and can be demonstrated over a broad range of fibroblast starting culture densities. Stimulation of fibroblast proliferation with serum and factor results in a synergestic response. Assays performed on fibroblasts attached to collagen substrates likewise demonstrate significantly increased fibroblast proliferation as a result of factor application. Other skin cell types, viz., keratinocytes and endothelial cells do not proliferate in response to secondary MLR supernatants. Also rabbit fibroblasts, rat epidermal cells, and human T lymphocytes continuously grown in TCGF (TL-2) do not respond. Absorption experiments show that both living and formalinized human dermal fibroblasts remove proliferative activity in dose dependent fashion from active supernatants. Experiments with non-responder cell types are in progress.

Significance to Biomedical Research and the Program of the Institute:

The major significance of this work is the elucidation of possible mechanisms of fibrosis occurring in disorders of cell mediated immunity.

This work may also have important implications to our understanding of systemic sclerosis, an idiopathic disorder of presumed autoimmune nature with dermal histology strikingly similar to that seen in chronic GVH disease. Finally, this work may ultimately provide insights into the regulation of collagen biosynthesis.

Proposed Course of the Project:

We plan to: 1) further characterize and purify the above mentioned factor with the ultimate goal of a) obtaining its molecular sequence and b) producing a hybridoma Ab specific for the factor. Essential to these goals will be the discovery of a tumor cell line that makes large amounts of this factor or hybridization of a producer cell with a non-producer myeloma line; 2) continue absorption studies with the ultimate goal of performing radiolabeled receptor binding assays. It remains to be demonstrated that cells not responding to factor lack a surface receptor for the factor; 3) characterize the serum factor that synergizes with the MLR SN factor to stimulate fibroblast proliferation. An examination of cell cycle kinetics in the presence and absence of factors may be informative in this regard; 4) set up an animal model so that in vivo experiments with purified factor and anti factor Ab's can be conducted; 5) begin experiments examining the effect of factor on collagen biosynthesis in an attempt to separate signals that stimulate cellular division from those that trigger product biosynthesis; and 6) examine serum and urine of patients with chronic GVH disease for the presence of factors causing excess fibroblast proliferation and collagen biosynthesis.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AI-00035-07 LI

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Specificity in Immune Responses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator:	John K. Inman, Senior Investigator, LI/NIAID
Other Investigators:	Dr. Suresh B. Shukla, MARC-NIH, Postdoctoral Fellow, LI/NIAID Dr. Ettore Appella, LCB/NCI Dr. Garrett C. DuBois, LCB/NCI Dr. Bruce Merchant, BOB/FDA Dr. Ronald Schwartz, LI/NIAID Dr. Daniel Hansburg, LI/NIAID Dr. Joyce A. Schroer, LMI/NIAID Dr. Kathryn E. Stein, LI/NIAID Dr. Collette Kanellopoulos-Langerin, LMI/NIAID

COOPERATING UNITS (if any)
Laboratory of Cell Biology, National Cancer Institute; Immunohematology Branch, Division of Blood and Blood Products, Bureau of Biologics, FDA; Laboratory of Microbial Immunity, NIAID.

LAB/BRANCH
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SECTION

INSTITUTE AND LOCATION
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The principal aim of this project is to test the hypothesis of general multispecificity for antibody combining regions and other types of receptors. Receptor sites should be capable of binding an occasional disparately structured ligand with an association constant large enough potentially to affect biological function. Radiolabeled antibodies or receptor-bearing molecules will be screened for multispecific binding to many, large haptens each bound to a separate affinity column matrix. Large haptenic reagents have been synthesized for this purpose using methods of peptide synthesis. Monoclonal antibodies from myelomas and hybridomas are being studied first. Multispecific bindings will be studied further by quantitative affinity chromatography.

Collaborative studies bearing on issues of specificity in immune responses have been and are being carried out; they deal with molecular interpretations of cellular mechanisms in immune responses. Chemical methods such as heteroligation and use of heterobifunctional reagents are employed in preparing specially designed antigenic materials, test reagents, and polyfunctional probes.

Project Description

Objectives

The overall objective of the project is to gain more complete understanding of the molecular basis for specificity in immune responses. The binding properties of antibodies and other receptor-bearing molecules are to be studied and related to the behavior of immune systems. Specific objectives are the following:

1. To assess the generality and frequency of multispecific binding by individual antibody combining regions by means of screening experiments employing many diverse, structurally unrelated haptens.

2. To develop methodology required for such screening tests including the synthesis of numerous, large haptenic reagents.

3. To employ multispecific binding and cross-stimulation in raising high titers of highly restricted antibodies for fine specificity studies and for use as highly specific reagents in immunological studies.

4. To use the large hapten screen to find disparate structure cross-reactions useful in identifying antibody-secreting cell clones in studies assessing antibody diversity.

5. To use the screen in studying the multispecific character of other types of receptors and to employ observed cross-reactions in a general approach to affinity separations of receptors.

6. To employ methods and reagents developed in this project in collaborative studies dealing with specificity in immune responses.

Major Findings

Work has continued on the synthesis of haptenic compounds required for the screening of antibodies for multispecific binding. These substances are branched amino acid and triazine derivatives which possess widely varied terminal structures but can be synthesized by common strategies and tactics. Each compound has a functional group that allows for covalent attachment to carriers and adsorbents. So far, we have prepared 20 derivatives of L-lysine and 12 of s triazine and 2 of L-ornithine. Some of these derivatives have been adequately purified and have met analytical requirements for purity and identity; others are now in final purification. Purifications are being carried out, in part, in the Waters 500A preparative liquid chromatograph.

The new haptenic reagents along with an earlier set, will provide a sufficient number of such compounds for a preliminary screening program. Preparations are underway to link these haptens to polyacrylamide gel particles for use in microaffinity columns. Radiolabeled myeloma proteins will soon be checked for known binding characteristics. Various types of polyacrylamide beads were tested for required physical and stability properties, and the appropriate one was selected and put through preliminary functionalization.

Haptenic compounds based on L-lysine have been prepared with an adipic acid residue linked td the side-chain amino group. The resulting terminal carboxyl function was modified as a free or protected hydrazide for later conversion into a reactive acyl azide. These syntheses were carried out by Dr. Shukla, and an outline of the strategy used has been reported (see publications). During the past year, Dr. Shukla has found convenient pathways to s-triazine-based haptens by which two or three of the chlorine atoms of 1,3,5,-trichlorotriazine (cyanuric chloride) are replaced by nucleophilic substances, one of which is a pre-assembled spacing arm with terminal, protected hydrazide for attachments. The most favorable order for assembling the triazine haptens was worked out; many of the intermediates and final products were crystallized after preliminary silica column purification. Dr. Shukla will present this work in a poster session at the next national meeting of the American chemical Society in San Francisco (Aug. 1980).

During the past year we developed the synthesis of a previously unreported reagent for introducing the 2-(4-tolysulfonyl)ethoxycarbonyl (Tsc) protecting group into amino acids and peptides. This effort arose from our need for an alkali-labile blocking group for amines that would be less polar than the previously employed methylsulfonyl (Msc) or ethyl (Esc) analogs. The Tsc group should impart to its derivatives improved crystallizing/chromatographic properties. Recentlu, we have been exploring more efficient means for the covalent attachment of haptens than is possible with amide bond-forming reactions, especially in aqueous media. Very promising results are being obtained with some newer strategies for the formation of sulfide (thioether) and disulfide linkages. Together, the above developments should greatly improve the next haptenic reagents prepared for this project in regard to theri design, use and synthesis.

The review that I wrote on peptide synthesis with minimal side-chain protection for Vol. 3, chapter 6 of "The Peptides" (see Publications) was carefully updated prior to final typesetting, an effort worth mentioning because of the considerable time required. This volume should be in print and available very soon. Also, I completed writing a chapter (with Dr. Appella, NCI) on peptide sequencing methology for a book to be published in England, edited by A. Darbre and M. Waterfield (see Publications).

In march of this year Prof. Richard N. Perham from Cambridge University, England visited me and Dr. Appella and his group. During the previous year we had collaborated in testing an idea which I proposed for the rapid cleavage of amidine linkages to peptide amino groups. Fairly extensive use has been made of acetimidyl groups and bis-amidine cross-linking in structural studies of proteins (primary, tertiary and quaternary), and, more recently, in protein semisynthesis. The ability to cleave amidine linkages can greatly extend their usefulness for such studies. Heretofore, these bonds have been broken by prolonged treatment of the peptide or protein derivative with concentrated aqueous ammonia. We tested, instead, the use of aqueous solutions of methylamine, a stronger nucleophile, and found an intrinsic cleavage rate that was 24-fold faster than that for ammonia. Amindine links and acetimidyl groups (with a ^{14}C tag) can thereby be split under considerably milder conditions without sacrificing the important technical advantage gained in removing a highly volatile reagent. The results of these experiments, designed by Dr. Perham and myself and carried out by Drs. DuBois and Appella, are described in a submitted manuscript and were recently presented at the International Conference on the Chemical Synthesis and Sequencing of Peptides and Proteins held at the NIH (May 8-9, 1980).

Another topic discussed during Dr. Perham's visit was the use of imidoester functions in the design of heterobifunctional reagents. These substances are useful in selectively cross-linking non-identical molecules without linking together identical ones. This strategy, called heteroligation, has many potential uses in basic and molecular immunology. Thus, it yields greatly improved means for preparing hybrid antigens, haptenating or sensitizing red cells, preparing enzyme-antibody complexes (for ELISA), studying membrane protein and receptor associations, etc. During the course of these discussions, I carefully reviewed this area of bio-organic chemistry and found that, by combining various approaches, many new improvements in heteroligation techniques would be possible. These ideas are currently being explored and tested in my multispecificity studies (see above in regard to sulfide and disulfide hapten linkages) and also in most of my collaborative work.

New heteroligation techniques are being employed in a collaborative study with Drs. Ronald Schwartz and Daniel Hansburg (LI/NIAID). Protein carriers have been thiolated by conventional and recently developed reagents. One of the latter, SPDP, was introduced by Dr. Jan Carlsson of Stockholm, and its method of synthesis was recently further improved in my lab. It should find extensive use in these and other studies. Further, we are using a reagent for introducing bromoacetamido groups that I designed and prepared several years ago. Together, these tactics have allowed conjugates of cytochrome peptides and carrier proteins to be cleanly prepared. Successful immunizations have been carried out using the conjugates, and the results will aid in studies of B- and T- cell function.

Heteroligation techniques have been employed in other collaborative studies. The use of a commercially available heterobifunctional reagent for sensitizing test red cells with mouse or human insulins allowed the development of an extremely sensitive and reliable assay for studying anti-insulin antibodies (cf., J. Schroer et al. under Publications). It is now possible to materially improve upon this method with newer heteroligating tactics. Currently, newer such techniques are being employed in collaborative experiments with Dr. Bruce Merchant and his group (BOB/FDA) in studies of B-cell differentiation.

Finally, some joint studies with Drs. Bruce Merchant and Harm Snippe (Utrecht, The Netherlands), involving the design and preparation of special hapten conjugates, were completed and papers have been submitted, some of which are in press. These studies concerned the B-cell defect of CBA/N mice and specificity in delayed-type hypersensitivity. An easy method for preparing DNP-red cell conjugates for studies of immune responses to the popular dinitrophenyl hapten will be reported by Dr. Kathryn Stein (LI/NIAID) et al. in the Journal of Immunological Methods (paper in press). This method grew out of a joint effort between myself and Drs. Stein, David Cohen (LI/NIAID) and Colette Kanellopoulos-Langevin (LMI/NIAID).

Significance to Biomedical Research and the Program of the Institute

An essential characteristic of many immune mechanisms is the specificity of action which is directed by definitive chemical structures. An understanding of specificity in immune systems rests first upon an adequate knowledge of the scope and nature of specificity at the level of single receptor-determinant interactions. Therefore, a principal aim of this project is to carry out the first systematic and general exploration of the phenomenon of multispecific binding. Because of the diversity and availability of antibodies, these receptor-bearing molecules provide ideal models for such studies in addition to their being interesting as important components of many types of immune reactions. Receptor multispecificity may play a fundamental role in drug action and side effects and in toxicological reactions.

Cooperative studies have proven the value of large haptenic reagents and other synthetic or semi-synthetic antigenic components in exploring specificity as it is manifested in more complex, multi-receptor-determinant interactions. Reagents or reactions developed for the explicit requirements of this project can have interesting applications in many basic studies in immunology such as those aimed at elucidating the mechanisms of B- and T-cell specific activation. Techniques for heteroligation that are employed and further developed in these studies can have many important applications in areas such as manipulation of immune responses and in the preparation of clinically useful test materials as, for example, specifically sensitized red cells and ELISA reagents.

Future Course of the Project

Details of the binding measurements are being worked out, and the systemic testing of monoclonal immunoglobulins will soon commence. A principal source of the latter will be hybridoma antibodies donated by other laboratories; about 1 mg of each one, purified, should be adequate for screening purposes. The rapidly increasing availability of hybridoma antibodies makes the present a very propitious time for beginning this phase of the study. Synthesis of large haptenic reagents will continue on a more-or-less routine basis in order to enlarge the repertoire of the screening program.

The future course of the project will the directed toward the following specific objectives:

1. To assess the occurrence frequency of various binding energy ranges of antibodies and to compare results with expectations from theoretical models.

2. To test other ligand-binding proteins (e.g., lectins, transport proteins and soluble receptors) for multispecific behavior.

3. To use the testing facility for locating some diverse specificities of antibodies, etc. for use in affinity purification and in functional studies in various systems.

4. To use the testing facility to develop disparate specificity profiles as clonal markers in studies of clonal expression control.

5. To use the testing facility for finding disparate specificities for anamnestic cross stimulation of selected clones to produce restricted antibodies.

6. To study thermodynamic and structural aspects of multispecific interactions for gaining a better understanding of multimodal binding.

7. To develop theoretical models for explaining selectivity, specificity and reliability in systems involving components with multispecific binding sites.

Collaborative studies will continue where chemical methodology developed and used in the above studies is applied in various areas of basic and applied immunological research.

Publications

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Snippe, H., Merchant, B., Lizzio, E., and Inman, J.K.: Functional antigen binding by the defective B cells of CBA/N x C3H/HeN F_1 male mice. J. Immunogenetics. In press.

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Honors

Advisory Editor to the journal, Molecular Immunology (term continuing).

Invited speaker at the International Conference on the Chemical Synthesis and Sequencing of Peptides and Proteins held at NIH on May 8-9, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z 01 AI 00036-15 LI

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)
Immunoglobulin Genetics: Regulation of Gene Expression and Lymphoid Differentiation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator: Rose G. Mage, Ph.D., Senior Investigator, LI/NIAID

Other Investigators:
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C. B. Alexander, LI/NIAID
G. O. Young-Cooper, LI/NIAID
H. B. Abdi, Visiting Fellow
A. Pavirani, Visiting Fellow
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COOPERATING UNITS (if any)
E. Appella, LCB/NCI; R. Asofsky, LMI/NIAID; F. Blattner, U. Wisc.; A. Chersi, Rome; M. D. Cooper, U. AL.; F. Finkelman, USUHS; T. Kindt, LIG/NIAID; T. Mosmann, U. Alberta; F. Mushinski, LCB/NCI; J. Rejnek, Prague; I. Scher, NMRI.

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

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SUMMARY OF WORK (200 words or less - underline keywords)

Overall goals of this project are to define the nature of genes which code for, or otherwise affect the structure of immunoglobulins (Igs) and to understand the processes which regulate Ig-gene expression and lead to synthesis of highly specific antibody molecules. We prepare immunological reagents which detect structural differences between genetically controlled polymorphic forms of rabbit Igs (Ig allotypes). We use immunological, chemical and molecular genetic approaches to define the Ig-structural differences and to learn more about the chromosomal organization of Ig genes. The regulated expression of antibodies, and antigen-specific receptors on lymphocytes. Inheritance patterns and the levels of regulation of phenotypic expression of alleles and chain types are investigated by breeding experiments, by studies of ontogeny and differentiation of cells of the rabbit immune system and by direct molecular genetic analyses. We use recombinant DNA technology to examine genome organization by isolation of rabbit Ig genes and to prepare cDNA copies of Ig mRNA. We plan to identify and analyze Ig variable and constant region DNA sequences as well as adjacent sequences and to characterize nuclear RNA precursors and their processing steps in the production of Ig mRNA.

Project Description

Objectives:

The objectives of this research program are to define in molecular terms, the organization and regulated expression of rabbit immunoglobulin genes, the levels of regulation of lymphoid cell differentiation, and the mechanisms which lead to the synthesis of highly specific antibody molecules and antigen-specific lymphocyte cell surface receptors. We seek a unified picture of the levels at which expression of Ig genes can be affected, and a detailed molecular description of the mechanisms which lead to the regulation.

Methods Employed:

For these studies, we prepare and characterize antisera which detect structural differences between genetically controlled polymorphic forms of rabbit Igs (Ig allotypes) and between classes and subclasses, types and subtypes of their polypeptide chains. We use immunological and chemical approaches to define the structural differences recognized by our antisera. We conduct both classical and molecular genetic experiments to learn more about the chromosomal organization of genetic information for the structure and regulated expression of Ig molecules. Problems which until recently have been approached at the levels of serology and classical Mendelian genetics are now amenable to direct molecular genetic analysis. We have started to clone rabbit genomic DNA, to prepare mRNA from cells producing rabbit Igs and to clone complementary DNA copies of the Ig mRNAs so that we can examine the organization of rabbit Ig genes and identify the nuclear RNA precursors and processing steps in the production of Ig mRNA. We breed and maintain colonies of rabbits with defined Ig allotypes and pedigrees for use in these studies. The rabbits and reagents we produce are made available to numerous other investigators at the NIH and are shipped in cooperation with the NIAID Research Resources Branch to investigators in laboratories throughout the U.S. and around the world.

Major Findings:

Ontogeny, gene expression, and lymphoid cell differentiaton

Our recent investigations of rabbit lymphod cell differentiation have included studies of the ontogeny of rabbit pre-B and B cells, the phenomena of allelic exclusion and alllelic imbalances in the expression of a and b allotypes in normal and allotype suppressed heterozygous rabbits. We are also investigating the nature of the defect in Bas mutant rabbits which leads to their production of serum Igs which are almost entirely of lambda light chain type. We conducted a thorough and systematic search for V_H structures which may be associated with membrane receptors of rabbit T lymphocytes. We hoped that rabbit T cells might provide a source of receptor material for biochemical characterization (Wilder et al. 1979,1980).

Florescent-labeled anti-allotype reagents have permitted studies of allotype expression in pre-B and B cells. They are providing informtion about B cell ontogeny and the stages of expression of heavy chain (V_Ha) and light chain (kappa, b) allotypes in heterozygous normal and allotype-suppressed rabbits.

Pre-B cells in developing rabbits were identified by immunofluorescence as cells containing small amounts of cytoplasmic IgM but lacking surface immunoglobulin. In the rabbit as in man and mouse, there are large pre-B cells which produce IgM (μ) heavy chains only. However, some small pre-B cells produce light chains. The pre-B cells in heterozygous b^4b^5, b^4b^9, and b^5b^9 rabbits in which we could detect these kappa chain allotypes appeared to exhibit allelic exclusion. These studies used affinity-purified, cross absorbed anti-b4, anti-b5 and anti-b9, all raised in b^6b^6 rabbits. The allelic imbalances seem at the B cell level in these heterozygotes were less marked or absent at the pre-B cell level. The preferential production of the b4 type compared to b5 and b9, and the b5 type compared to b9 in these heterozygotes thus appears to be due to the expansion of a greater proportion of one type of allelically excluded B cell compared to the other, rather than because of preferential differentiation for expression of one chromosome compared to the other at an early pre-B cell stage.

V_H-bearing immunoglobulin has also been detected in rabbit pre-B cells using affinity-purified, cross-absorbed a1 anti-a2 and a1 anti-a3 allotype reagents and homozygous a^2a^2, a^3a^3, and heterozygous a^2a^3 rabbits. The majority of cells with cytoplasmic IgM have detectable V_Ha allotypes (56-100%). Essentially all of the pre-B cells which are detectable with our fluorescent anti-allotype reagents exhibit allelic exclusion of the a allotypes. Again, allelic imbalances seen at the B cell level are less marked or absent at the pre-B cell stage.

Although Bas mutant rabbits produce Igs which are almost entirely of the lambda light chain type, they produce traces (of the order of 1 μg per ml) of Ig with kappa type light chains detectable with anti-b9 reagents. In collaboration with Dr. M. D. Cooper and W. E. Gathings we have shown that pre-B cells with b9-like kappa chains are detectable in b4-bas, and b5-bas adult bone marrow and in b4-bas and bas-bas newborn marrow. The exact quantitation is still in progress, but the proportion of pre-B cells with detectable b9-like light chains in the heterozygous b4-bas and b5-bas adults appears to be greater than the subsequent proportion of B cells bearing the b9-like kappa chains.

We studied rabbit PBL by anlytical flow microfluorometry (FACS analysis in collaboration with Dr. I. Scher). Doubly heterozygous rabbits were used and single populations of cells were scored for proportions of total Ig-, b4-, b5-, a2-, and a3-positive cells. The proportions of Ig^+ cells when compared to the (a2 + a3) positive or (b4 + b5) positive cells gave no evidence for B cells expressing the two allelic allotypes (Abdi et al. 1980). Because some published reports of allelically included cells used a rosetting assay, results of FACS analysis and rosetting were compared on the same cell preparations. Rosetting assays were not found to be more sensitive than the FACS for detection of b-allotype-positive PBL of homozygotes. However, an excess of b4 + b5 positive cells over total Ig cells (5-25%)

was seen in some heterozygotes' cell preparations. Although some of these cells may be truly allelically included, we believe that some are the FcR positive non-lymphocytes now seen by other including Gathings et al. and Cabana et al. (1980). Our cellular and serological studies of allelic exclusion have led us to conclude (Abdi et al., 1980) that definitive proof that any allelically included cells exist will require their purification, isolation of their DNA and analysis by molecular genetic techniques to determine whether the V and C genes are in a re-arranged form compatible with expression of the allelic products of both parental chromosomes. In studies of allotype-suppressed rabbits of b4b5 allotype, we found that pre-B cells of the suppressed allotype persist when B cells of that allotype are absent. The persistence of pre-B cells of the suppressed type supports the view that pre-B cells differ in their responsiveness to external influences such as anti-Ig compared to B lymphocytes. Surviving pre-B cells are a likely source of the B cells which appear within 24 hr of administration of neutralizing antigen to 14-23 day old suppressed heterozygotes as well as for B cells of suppressed allotype which appear during the recovery phase of allotype suppression (Simons et al., 1979). We have found that cells bearing sIg of the suppressed allotype appear and increase toward normal proportions in rabbit PBL even when serum Ig levels of the suppressed type are markedly depressed (Simons, M., Ph.D. thesis, 1979 and Abdi, Scher and Mage, in progress).

A hypothesis was advanced that rheumatoid factors produced by some individuals are reflections of exaggerated production of normal regulatory anti-globulins analogous to the regulatory idiotypic network described by Jerne. The specificity of such factors may not, however, be for idiotypes, but for allotypes or other characteristic antigenic determinants on Igs. We used sera from allotype-defined rabbits, and human sera containing rheumatoid factos to investigate this question. Isolated rabbit and human rheumatoid factors did not distinguish between specific rabbit C_H allotypes of IgG (Wilder, DeRito and Mage, 1979) presented at the American Rheumatism Association, Southeastern Regional Meeting, December 1979.

Antigen-specific surface receptors of B and T cells

Staphylococcal protein A and several different immunoglobulins were radiolabeled to high specific activities (10^6 cpm/μg) by reductive methylation with tritiated solium borohydride. The proteins retained excellent functional and antigenic properties. The reagents were used in a variety of assays for cell surface antigens as well as solution immunoassays. This radiolabeling procedure has become the method of choice for many cell surface and solution immunoassays currently being performed in the Laboratory of Immunology (Wilder et al., 1979a). T and B lymphocytes appear, at least partially to share idiotypic determinants. Sharing of V_H framework determinants is more controversial. In order to examine this question, we have prepared heterologous anti-rabbit and anti-mouse V_H antisera. Considerable characterization has been done of these reagents (Wilder et al. 1980, and work in progress) and the sera are being used to further examine the T cell receptor question (Wilder et al., 1979b). In addition we have supplied the anti-rabbit V_H and the anti-mouse V_Hu to a number of investigators for further testing immunochemically and in cell functional assays. In one study (Taniguchi, M., personal communication) the anti V_Hu antiserum removed the antigen-specific suppressor produced by a T cell hybridoma.

We have studied the extent to which V_H framework determinants are represented on T cells in the rabbit using anti-a (V_H framework) allotype sera and anti-rabbit V_H sera made in goat (Wilder et al., 1979b,1980) and chicken. Flow microfluorometric analyses of splenic lymphocytes from hyperimmune rabbits demonstrated closely similar frequencies of cells stained with the anti-V_H framework antisera compared to anti-light chain antisera. To search for potentially hidden molecules, additional studies were performed with radioiodinated, detergent solubilized plasma membrances derived from immune spleen and lymph nodes. No evidence for a population of cells or a class of molecules(s) bearing V_H in the absence of light chain constant region determinants was obtained (Wilder et al., 1979b). One possible interpretation of our data is that only a small T cell subpopulation expresses V_H determinants. We also have not ruled out the possibility that T and B cell receptors have V_H structures with only limited antigenic similarity. Our rabbit T cells have not provided a source of material for biochemical characterization of T cell receptor.

Structure and allotypes of rabbit immunoglobulins

Most of the amino acid sequence of the a3 heavy chain variable region (Appella and Mage) and the complete sequence of the b4 light chain from anti-SIII antibody AH80-5 has been completed. The light chain sequence is of interest because of its great similarity through position 90 with a minor light chain subgroup frequently expressed by rabbits responding to hyperimmunization with the A variant strain of Streptococcus, and with *Micrococcus lysodeikticus*. The rabbit kappa-type light chain has been considered unusual because it does not appear to have as marked hypervariability between positions 50 and 60 within which the second hypervariable region of human and mouse kappa chains has been defined. This particular rabbit light chain has an insertion within this portion of the sequence (Chersi et al., 1979).

The partial sequence (positions 29-17) of the variable region of light chains of predominately b5 allotype from the IgG of a single allotype-suppressed rabbit was obtained by traditional sequencing methods on isolated tryptic and chymotryptic peptides. The peptides from this region were isolated in relatively high yields and probably represent a dominant sequence. The framework sequence between positions 35 and 49 (FR2) is identical to an FR2 sequence commonly found in antibodies produced by b4 rabbits as well as in murine and human myeloma light chains, with the exception of an interchange of threonine for proline at position 43 or 44. They may be b5 allotype-related since to date all b4 light chains have had proline and a b9 light chain was found with arg at position 43. The fact that a dominant sequence could also be found for positions corresponding to the second CDR (50-56) in other species, confirms previous observations that this portion of the light chain is not extremely variable in the rabbit (Chersi, Mage and

Alexander, 1979). Peptides containing tryptophan 146 from rabbit kappa light chains of b5 and b6 aallotypes were isolated and their sequence determined. Between positions 138 and 161 there is 56% homology between b4 and b9. Between positions 138 and 150 there is 86% homology between b4 and b6 and 93% homology between b5 and b6. This confirms our predictions from earlier serological studies (Landucci Tose et al., 1975) that b9 light chain constant regions would differ most from the other allotypes, while b5 and b6 would be most similar in sequence (Chersi and Mage, in press, 1980). The sequence of the entire constant region of b5 light chains is almost complete (Chersi, Alexander and Mage, in press, 1980). We find that b5 is about 80% homologous with b4 and 60% homologous with b9. This sequence data is important to us now as we begin to search for genomic recombinant DNA clones containing the coding sequences for rabbit kappa type light chains.

Breeding Experiments and Investigations of Chromosomal Organization

New data on the structure of Ig genes at the DNA level stimulated a re-evaluation of a number of questions concerning the arrangement and rearrangement of genes for rabbit Ig-heavy chains. A tabulation of the postulated heavy chain J-coding sequences from IgGs of known a-allotypes showed no apparent correlates of sequence and a-allotypes for this portion of the V_H domain (Mage, 1979,1980). A tabulation of postulated J-coded sequences of rabbit kappa light chains predicts that these may be 8-9 different J segments found for light chains of b4 allotype (Mage, 1980). Another general question raised was whether the apparent recombinations observed by ourselves and others within the heavy chain linkage group were intra-V_H, between V_H and J, intra-J, between J and C_H or indeed several of these. Some recombinations in the H-chain genetic region may be undetectable or poorly detectable because the recombinations result in disruption of mechanisms for efficient gene reorganization. Occasional, although infrequent, expression of certain genes after such recombinations might then lead to observation of "hidden or latent allotype or idiotype" production (Mage, 1979,1980).

Recombinant DNA technology has permitted a detailed examination of the molecular organization and expression of mouse immunoglobulin genes. Molecular biological studies in the mouse system have led not only to a much better understanding of how immunoglobulin genes function but also to a greater awareness of the potential complexity of other eukaryotic gene systems. As we begin to apply similar techniques to an analysis of the rabbit system, we anticipate that some findings will be similar to those in the mouse but that others may be quite different.

We have begun to analyze the rabbit immunoglobulin system at the molecular biological level. Our primary objectives have been twofold: 1) to isolate, identify, and analyze the mRNA which is the gene product of the rabbit immunoglobulin genes, and 2) to isolate, identify, and analyze the genes which encode these mRNA products. We anticipate that when these two objectives have been met, we will be able to better analyze the regulation of the expression of these genes at the molecular level. Such further analyses of regulation of expression are anticipated to include both studies of gene rearrangements during immunoglobulin gene expression and studies of nuclear RNA transcription and processing.

We have prepared mRNA from the spleens of hyperimmunized rabbits and demonstrated that it is physically intact when subjected to methyl mercury hydroxide-agarose gel electrophoresis. The biological activity of individual RNA preparations has been demonstrated in a cell-free translation system and compared with the biological activity of spleen cells derived from the same rabbit. We are confident that we have isolated mRNA which encodes rabbit light chains as well as rabbit heavy chains.

We are using these mRNA preparations to prepare cDNAs and thence recombinant DNA molecules containing a single rabbit light chain or heavy chain. These recombinant DNA molecules will provide source material for an analysis of mRNA structure and nuclear RNA transcription and processing as well as probes fro studies of genome organzation.

To analyze the organization of rabbit immunoglobulin sequences in the genome, we have embarked upon genomic cloning studies using vectors provided by Dr. Fred Blattner. We have prepared high molecular weight DNAs from rabbit somatic tissue (spleen and liver) and from rabbit germ line material (sperm). These DNAs included the following allotypes: a1, a2, a3, b5, b9, bas, e14, and e15. In collaboration with Drs. Fred Blattner and Chih-ping Liu, we have constructed genomic libraries from one sperm DNA preparation and from two spleen DNAs. These libraries contain as many as fifty genome equivalents of DNA. We are in the process of screening these libraries to identify and isolate genes of interest for further characterization. We also intend to construct other libraries so as to identify and isolate other genes.

Screening of these libraries currently is being done using characterized clones containing mouse immunoglobulin light and heavy chain sequences. We anticipate that the recombinant cDNA probes will greatly aid in the rapid screening of these genomic libraries.

We also are conducting two studies of the cross-hybridization of mouse and rabbit immunoglobulin sequences. Rabbit sperm DNA has been prepared, digested with various restriction endonucleases, subjected to agarose gel electrophoresis, and blotted on DPT paper. Several mouse Ig H and L chain probes have been shown to hybridize to DNA fragments on these blots. These studies are being extended using the additonal techniques of RPC5 column chromatography.

Similar cross-hybridization studies are in progress using blots of rabbit mRNA from methyl mercury hydroxide-agarose gels. Again, several mouse probes have been shown to hybridize to mRNA species fractionated on these gels.

We are also conducting experiments in several mouse myeloma systems: TEPC 609, TEPC 1033, and TEPC 1017. The studies in the TEPC 609 system were initiated while Dr. Fitzmaurice was a fellow in Dr. Randolph Wall's laboratory of UCLA and are being continued in collaboration with him and with Dr. Tim Mosmann. The double-producing mouse myeloma tumor, TEPC 609, was adapted to tissue culture and cloned by Dr. Mosmann and shown to produce simultaneously a γ_{2b} and an α heavy chain as well as a λ light chain. In studies conducted by Dr. Mosmann and Dr. Fitzmaurice in Dr. Wall's laboratory at UCLA, the messenger RNAs encoding the heavy chains were examined on methyl mercury hydroxide gels and shown to be similar in size to the γ_{2b} heavy chain mRNA produced by cloned cell line 45.6 and the α heavy chain produced by cloned cell line 460.1. Examination of nuclear RNA transcripts and processing intermediates on methyl mercury hydroxide gels indicated that the messenger RNAs for these two TEPC 609.8 heavy chains are transcribed simultaneously. The primary nuclear RNA transcript for the γ_{2b} heavy chain was found to be similar in size to that identified for the α heavy chain. We have been investigating the possibility that these two heavy chains are processed from the same primary nuclear RNA transcript.

Dr. Fitzmaurice also is involved in a series of collaborative experiments directed towards an understanding of the transcription and processing of mRNA encoding mouse IgD. These experiments are being done in collaboration with Dr. J. Fred Mushinski, Laboratory of Cell Biology, NCI, who, working with Dr. Phil Tucker and Dr. Fred Blattner, constructed a cDNA clone containing δ sequences from TEPC 1C17. Dr. Fitzmaurice and Dr. Mushinski have isolated RNA from mouse myelomas TEPC 1033, TEPC 1017, and lymphoma line BCL-1 and from mouse spleen, analyzed these RNAs on methyl mercury hydroixide gels, and identified RNA species containing δ coding sequences. They also have isolated and analyzed RNA from several IgM-producing cell lines. A detailed analysis of the transcription and processing of δ mRNA and its relationship to the transcription and processing of μ mRNA will provide an answer to the question of whether these two heavy chains are co-granscribed. Further, analysis of the transcription and processing of what appear to be multiple cytoplasmic δ mRNA species will aid our understanding of how the expression of   may be regulated.

Significance to Biomedical Research and the Program of the Institute

Immunogenetics continues to be at the forefront of our understanding of the nature of structural and regulatory genes in complex mammalian systems. As we increase our knowledge of the genetics and evolution of Ig genes and of the chemistry of Ig proteins and the DNAs which code for and regulate their production, the Ig system becomes one of the most valuable models for investigation and understanding of the functioning of complex genetic regions on chromosomes. These systems are providing new knowledge of the molecular basis for cellular differentiation in higher organisms. The interrelationships of the cell surface antigenic systems controlled by the major histocompatibility locus and the Ig gene systems are becoming increasingly apparent as knowledge of the two systems increases. The increased knowledge of Ig genetics provides models of direct relevance to understanding the evolution and genetic organization of cell surface antigenic systems. Understanding the chemistry, genetics and evolution of Igs, the factors which control their genetic expression and the differentiation pathways of the cells which produce them is of broad fundamental interest.

Proposed Course of the Project

Most of the projects described are in progress and continuing. Much of the evidence for linked inheritance and cis expression of V_H and C_H genetic information, allelic exclusion, imbalances in quantitative expression of "allelic" genes and "latent allotypes, or hidden genes" has come from the rabbit model. These phenomena continue to be studied intensively. In addition to our studies at the immunochemical, T cell, pre-B and B-cell levels, with the advent of recombinant DNA technology, a major new initiative in the laboratory has been to develop molecular genetic approaches to the problems which have previously been studied by the more classical immunogenetic approaches. We expect to prepare libraries of cloned recombinant DNA from rabbits of defined allotypes in addition to those already in hand, and to characterize clones containing genes of interest. In addition, we are undertaking cDNA cloning using reverse transcripts of mRNA preparations containing mRNAs for rabbit Ig chains of defined allotypes. We expect that the experiments in progress to identify and isolate structural genes for the H and L chains of rabbit Igs from differentiated and non-differentiated cells as well as to identify nuclear RNA precursors and their processing into Ig mRNA will considerably increase our understanding of the organization and regulated expression of rabbit Ig genes.

Appendix

Contract number N01 AI 82565-Maintenance and Breeding of Rabbits of Known Genotype and Their Use in Immunological Studies.

The objectives and methods are related to those outlined above. The contract has continued to provide outstanding support through production and maintenance of allotype-defined rabbits, immunizations, bleedings, and various short-term and long-term projects involving breeding, immunizations and suppression. A procedure for collection of rabbit sperm from sperm DNA donors was put into operation. Rabbits and reagents produced and supplied through this contract were made available to numerous other investigators at the NIH and were shipped to laboratories throughout the US and around the world.

Publications

Wilder, R.L., Yuen, C.C., Subbarao, B., Woods, V.L., Alexander, C.B. and Mage, R.G.: Tritium (^{3}H) radiolabeling of protein A and antibody high specific activity: application to cell surface antigen radioimmunoassays. J. Immunol. Methods. 28: 255-266, 1979a.

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Chersi, A., Mage, R.G. and Alexander, C.B.: Partial sequence of the variable region of normal rabbit IgG light chains of b5 allotype. *Molec. Immunol.* 16: 833-836, 1979.

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Abdi, H.B., Wilder, R.L., Scher, I. and Mage, R.G.: Surface immunoglobulin of rabbit peripheral blood lymphocytes: detection of allotypes by fluorescence and rosetting. *Eur. J. Immunol.* In press, 1980.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AI-00037-13 LI

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)
Immunogenetics of Mouse Immunoglobulins and Genetic Control of Antibody Response

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) Our research program is aimed at determining the genetic and structural basis of antigenic polymorphism of the constant region (allotypes) and variable regions (idiotypes) of antibodies and at the genetic and cellular levels, the effect of regulation on the idiotypes and allotypes associated with specific anti-hapten antibodies. The possibility that allelic structural genes are responsible for the polymorphism of the H-chain variable regions is being investigated for anti-phosphorylcholine antibodies. The expression of T15 Id associated with anti-PC antibodies and PC binding myeloma proteins of BALB/c and C3 Id with PC antibodies and binding myeloma proteins of C57BL/6 in backcross progeny and Ig congenic and recombinant inbred strains derived from these 2 strains suggest that these behave like allelic markers. IEF patterns of BALB/c IgG anti-inulin and anti-levan antibodies show that predictable restricted anti-inulin spectrotype patterns become more heterogeneous and complex in the presence of a regulator gene designated SR-1 contributed by C57BL/6. BALB/c (Igh-C^a) mice immunized with BL produce IgG anti-inulin antibodies of a single spectrotype. C57BL/6 (Igh-C^b) does not produce anti-inulin antibodies. (CxB)F_1 produce IEF patterns that are considerably more heterogeneous and complex than BALB/c suggesting a regulatory gene SR-1 contributed by C57BL/6. SR-1 is not linked to Igh, MHC, or coat color genes.

A. Allelic V Region Markers in the Mouse

The possibility that allelic structural genes are responsible for the polymorphism of the H-chain variable regions were investigated. Recently it was shown that the amino acid sequence of H-chain variable regions of 2 phosphorylcholine binding myeloma proteins T15 from BALB/c and C3 from C.B20 show complete homology except for 3 interchanges in the framework of amino acids. With antiidiotype antisera prepared to these proteins, we are able to show that each protein has an IdI that is also present and can be distinguished in normal anti-phosphorylcholine antibodies of BALB/c and C57BL/6 mice. The expression of these IdI e.g., T15 is linked to Igh-C^a and C3 is linked to Igh-C^b. Genetic studies of anti-phosphorylcholine antibodies from crosses of BALB/c (Igh-C^a) and C57BL/6 (Igh-C^b) and of Ig congenic and recombinant inbred strains derived from these 2 strains corroborate that the genes IgVh-PC^a and IgVh-PC^b which control the expression of T15 and C3 IdI respectively, behave like alleles suggesting the existence of polymorphism of V-region markers coding for H chains of anti-phosphorylcholine antibodies.

B. Igh-C and Igh-V Markers Studied in Wild Mice

Idiotypes linked to specific allotypes in inbred strains of mice and associated with anti-inulin and anti-phosphorylcholine antibodies and myeloma proteins that bind these respective haptens have been studied in the Mus genus comparing several species and subspecies of mice. These mice have been immunized with bacterial levan to produce anti-inulin antibodies and with Streptococcus pneumoneae R36 to elicit anti-phosphorylcholine antibodies. In addition Igh-C allotypes have been determined. Most wild mice exhibit allotypes similar to those of inbred strains. Some phenotypes found suggest recombination of C region genes. Some species especially those furthest in evolution from *M. musculus domesticus* strains show no allotypic markers found in inbred strains. A survey of anti-inulin and anti-phosphorylcholine antibodies of inbred and wild mice for Igh-V-InIdX and Igh-V-PC-IdI show a non-concordant expression of these markers with allotype. These studies are particularly important in mapping of V region genes and also in elucidating the genetic and structural basis of antigenic polymorphism reflected by the large diversity of antibodies produced.

C. Regulation of the Anti Inulin Antibody Response by a Non Allotype Linked gene Sr-1

The ability to make antibodies to inulin (B2→1 fructose) in mice immunized with bacterial levan (BL) is determined by Igh-V genes linked to specific Igh-C^a genes. These antibodies express a cross-reactive idiotype (IdX) also found on inulin binding myeloma proteins. Isoelectric focusing of BALB/c IgG anti-inulin antibodies show that these are highly restricted and give a characteristic spectrotype consisting of 3 major and 2 minor bands. This characteristic spectrotype is absent in serum of C57BL/6 mice immunized with BL. CBL/6 mice for the most part only produce anti β2→6 and not β2→1 (inulin) antibodies. The IEF pattern of IgG anti BL antibodies of BALB/c x C57BL/6 F_1 mice is much more complex and heterogeneous than BALB/c and contained several bands in addition to the characteristic band patterns of BALB/c. Ig congenic strain B_aC8 and recombinant inbred strains CxBJ and CxBG which are of the Igh-C^a haplotype also showed this heterogeneous spectrotype pattern. These findings suggest that while the production of IgG anti-inulin requires the Igh-C^a allotype, the complexity of the IEI pattern is regulated by a C57BL/6 gene. Analysis of backcross progeny suggest that a spectrotype regulatory gene designated Sr-1 which is not linked to Igh, MHC or coat color genes is responsible for the increased complexity of the response. These findings are particularly important in pointing out that while structure Igh-C and Igh-V genes are essential in determining the synthesis of a particular antibody, the expression of these antibodies are markedly affected by other genes (regulatory) not linked to the Ig system.

D. Studies of Inheritance and Expression of γ_{2a} and δ Allotype in Backcross Generations of (C57BL/6 x DBA/2) x C57BL/6 Progeny

(C57BL/6 x DBA/2) x C57BL/6 progeny were produced in the course of studies to determine linkage of the B lymphocyte differentiation markers Lyb 7 to γ_{2a} and δ loci. Two of the mice exhibited IgD but not IgG_{2a} allotypes of DBA/2. These 2 putative recombinants were bred to C57BL/6. Progeny with $IgG_{2a}^{\ b}$ (C57BL) and IgD^a (DBA/2) were selected for each generation and backcrossed to C57BL/6. These progeny are being studied for: 1) possible recombination of C region genes and 2) possible regulation of the expression or suppression of the $Ig_{2a}^{\ c}$ allotype of DBA/2 by C57BL genes. Appropriate matings will be done to segregate "regulating" genes and/or "recombinant genes".

PUBLICATIONS:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AI-00038-08 LI

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Structure and Activity Studies on Immunologically Important Cells and Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Principal Investigator: Myron J. Waxdal, Ph.D., Sr. Investigator, LI/NIAID

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INSTITUTE AND LOCATION National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	2.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An understanding of the mechanisms by which lymphoid cells transfer information signals are the major goals of this project. These include the transfer of controlling or modulating signals between lymphocytes and also the biochemical pathways by which signals from outside the cell are transmitted past the cell membrane; from the receptors on the cell surface, passing thru the lipid bilayer, and finally redirecting the internal metabolism of the cell. The first of these studies extends our initial observations with a series of helper factors (LSF) produced by T and thymus cells in response to Pa-2 activation. The second extends our studies on membrane phospholipid methylation in response to Con A stimulation.

To facilitate these studies we are also seeking to identify and fractionate subpopulations of lymphoid cells: both the producers of each LSF and the targets of these intracellular signals. This is an extension of our studies of differential lectin binding to cells.

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(Rev. 10-76)

Intracellular Communication via Soluble Factors

Murine lymphocytes were stimulated with the pokeweed mitogen Pa-2, washed and placed in culture in the absence of added mitogen. Cell free supernatants were harvested at various times and assayed for their ability to stimulate DNA synthesis and the formation of IgM secreting cells. DNA synthesis was measured by thymidine incorporation and IgM secreting cells by a modification of the reverse plaque technique. Six separate fractions have been obtained, one of which appears to stimulate B cells in the absence of T cells.

This year our efforts have been directed at further purification and characterization of these LSF. An important finding was that LSF produced by murine thymus or splenic T cells is able to stimulate both DNA synthesis and immunoglobulin production by cultures of human peripheral lymphocytes. In identifying the subset of cells that produce LSF, Pa-2 has been successfully used to isolate the small (approximately 7%) subpopulation of thymocytes that produce LSF in response to this mitogen. It was also found that the murine thymus produces a different set of LSF than that produced by the spleen. There does appear to be a considerable overlap, however. In addition. the profile of LSF produced by the thymus appears to change as the animal ages.

Further biochemical and biological characterization of the individual LSF are planned. Initially this will be to determine the effect of LSF on specific antibody responses and to isolate and characterize the B cell stimulating material. Further exploration of the subsets of thymocytes and splenic T cells which produce each LSF and the cells which are the primary target of LSF activity are planned. These probably will involve the use of alloantisera and other lectins for the characterization and fractionation of the cellular subsets.

Biochemical Events in Transmembrane Signaling

The objectives of our research in this project is to determine the earlier (first few minutes) biochemical events and pathways which are important in the activation of B and T lymphocytes.

Previously, we eludicated a series of biochemical steps in the cell membrane using Con A to activate murine T cells. These include the successive methylation of phosphatidyl ethanolamine to form phosphatidyl choline (lecithin), followed by the degradation of lecithin to yield arachidonic acid and lysolecithin. Our current studies have shown that this pathway is also stimulated by several other mitogenic lectins, but not by non-mitogenic lectins. Furhtermore, in preliminary experiments we have also found that LAF on T cells and anti-μ chain on B cells both activate this biochemical pathway. Stimulation of cultured human T cell lines with PHA produced T cell Growth Factor (TCGH) also stimulate the same biochemical events. These observations suggest that this pathway is common to several different modes of lymphocyte activation.

The biochemical observations we have made on this system will be studied in further detail. The elucidation of the membrane events by which the binding of mitogenic lectins to the cell surface acceptors activates the transmethylase enzymes will be sought. The activation of the calcium dependent phospholipase A2 by mitogens will also be studied. One of the approaches planned to study these membrane associated enzymes is by raising antisera directed against the isolated proteins. These antisera will be fluoresceinated and used to detect changes in the distribution or availability of these enzymes on the cell surface during the activation.

The biochemical pathways will also be followed downstream in an attempt to link subsequent events (such as changes in the levels of cyclic nucleotides, protein phosphorylation, etc) to the currently delineated events. These studies will be pursued by the use of radioactive metabolites, specific inhibitors, and enzyme assays.

Lectin Fractionation of Lymphoid Cell Subsets

In collaboration with LMI/NIAID and LCI/NIAID we have extended to human tissue our studies on the preferential binding of approximately 40 lectins to subsets of lymphoid cells.

Lectins used in these experiments were either isolated by this laboratory, obtained from other researchers, or purchased commercially. In most cases the lectins were assayed for purity by polyacrylamide gel electrophoresis in sodium dodecyl sulfate, ion-exchange chromatography, and/or affinity chromatography. Lectins were fluorescein labeled and agglutination titers were determined for each cell type with each lectin. For each analysis fluorescence was measured on 50,000 viable lymphocytes by flow microfluorometry on a Fluorescence Activated Cell Sorter (FACS).

Several lectins were found which showed bi- or polymodal staining to human peripheral T cells, B cells monocytes, and to 'null' cells. It was noted that this group included lectins which gave only unimodal staining when tested on murine lymphocytes. Our studies on the identification of subsets of murine lymphocytes have progressed to the use of lectins to fractionate these subsets. We have explored several methods for the lectin fractionation of cells. These include but are not limited to: lectin affinity column chromatography, covalent binding of the lectin to culture plates, and covalent binding of glycoproteins to culture plates followed by affinity binding of the lectin to the glycoprotein. To avoid having to design a specific technique for each lectin, we are attempting to delineate a general procedure by which many different lectins may be individually bound to culture plates and used for the fractionation of lymphoid and other cells. At the same time, and dependent upon the development of fractionation techniques, we are characterizing the subsets by other cell surface markers. We have also initiated functional characterization of these fractionated subsets of murine and human lymphocytes by their biological activities. These include helper activity, production of LSF, and suppressor activities.

The future course of this project will be to emphasize the development of separation techniques for the subsets recognized by the lectins and to characterize the fractionated subsets of both human and murine lymphocytes.

Structure Studies on Guinea Pig Ia Antigens

Inbred strain 2 and strain 13 guinea pigs, and alloantisera to their Ia antigens were obtained from Dr. E. Shevach, LI. The Ia antigens were labeled by the metabolic incorporation of a mixture of ten radiolabeled amino acids, single amino acids, and sugars into spleen cells. The cells were lysed with NP-40 and the Ia antigens isolated by affinity chromatography on columns of lentil lectin followed by immunoaffinity chromatography or immune precipitation. The alpha and beta chains were isolated by polyacrylamide gel electrophoresis in sodium dodecylsulfate with and without prior reduction of the disulfide bonds. Amino acid sequence studies of the amino terminal regions have prevously been performed on the isolated chains. During this past year, we extended our studies of the homology and differences between the chains of the guinea pig Ia antigens to include internal regions thru the use of peptide mapping techniques.

Peptide mapping studies by ion-exchange chromatography of tryptic peptides of the alpha and beta chains from Ia.3,5 labeled with 10 amino acids showed that many of the peptides elute at the same positions. This suggested that there may be regions of major conservation of structure between the two polyppetide chains of the same Ia molecule. Further mapping, with single amino acid labels (tyrosine, arginine, lysine, tryptophan), has not shown nearly as much homology as suspected from the initial observations using 10 amino acids and has led us to focus our attention on specific regions of the chains. Labeling the carbohydrate moiety of each chain with glucosamine has shown that the alpha and beta chains each contain carbohydrate attached to one potential tryptic peptide. A complimentary approach has been to label the Ia chains with 10 amino acids and then to isolate the carbohydrate bearing tryptic peptides by affinity chromatography on Con A resins. The results of these two approaches have been essentially the same. Further studies by chymotryptic mapping of these carbohydrate containing tryptic peptides, have shown that they are very similar, suggesting that the amino acid sequences of the alpha and beta chains of the guinea pig Ia.3,5 molecule may be homologous in this region.

This project has been unfruitful in spite of a considerable investment of effort and expense. Two manuscripts are being prepared from the data obtained in these studies. There are no plans to continue this project.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-AI-00040-06 LI

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)
Genetic Control of Immunocompetent Cell Interactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any) Dr. Hinrich Bitter-Suermann, Dept. of Pathology, Georgetown University Medical School

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SECTION

INSTITUTE AND LOCATION National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.5	4	2.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long range goals of this project are to determine the role of I-region associated (Ia) antigens in the regulation of immunocompetent cell interactions and to determine the mechanism of action and site of expression of the histocompatibility linked immune response (Ir) genes. During the past year studies have been performed in three major areas: 1) we have demonstrated that the binding of large amounts of anti-antigen antibody to the macrophage surface results in marked inhibition of T cell proliferation and that this result is consistent with the view that "processed" antigen is displayed on the cell surface perhaps in association with Ia antigens; 2) using a model system in which T cell stimulation is induced by active aldehydes (CHO) on the macrophage surface, we have shown that Ia antigens play a critical role in CHO induced T cell proliferation and may function as triggers or second signals in this process; 3) we have shown that monoclonal antibodies to guinea pig Ia antigens react with different functional parts of Ia molecules and that certain parts of an Ia molecule participate in the presentation of certain antigens while other regions of the same molecule present different antigens.

I. Role of the I-Region in the Regulation of Macrophage-T Cell Interaction

Studies from several laboratories have demonstrated that it is exceedingly difficult to inhibit the interaction of antigen-pulsed macrophages with antigen specific T cells with high-titer antibody to antigen. It has therefore been suggested that the immunologically relevant antigen is in a site inaccessible to antibody, i.e. intracellular or perhaps buried in the lipid of the membrane. Alternatively, the T cell receptor and antibody to antigen may recognize distinct portions of a complex protein antigen.
Thus, the T cell receptor may recognize certain antigen fragments present on the macrophage surface in association with macrophage Ia antigens and the fragments that stimulate the T cell fail to elicit antibody production. A third possibility, most consistent with a rigid interpretation of the "altered self" theory, is that the T cell receptor fails to recognize any determinants of the native antigen, but recognizes an antigen-induced alteration of macrophage Ia antigens.

We had previously demonstrated that when T cell proliferation is induced by haptens coupled to the macrophage surface the binding of sufficient antihapten antibody to the macrophage surface leads to inhibition of T cell proliferation and that antigenic determinants are expressed on the surface of the antigen presenting cell. Although it is likely that the macrophage presentation of haptens coupled to the cell surface is representative of the macrophage handling and presentation of soluble protein antigens, we felt that it was crucial to extend our studies on the effects of anti-antigen antibody to T cell proliferation induced by macrophages pulsed with soluble protein antigens. We quickly confirmed the observations of earlier workers that high titer antibody to a variety of protein antigens had no effect on the T cell proliferative response. We therefore hypothesized that if antigen is present on the surface of the presenting cell and is capable of being labeled with TNP, sufficient anti-TNP antibody should bind to the antigen and to TNP-labeled membrane proteins to lead to capping and shedding of the antigen from the cell surface. Macrophages were pulsed with several protein antigens [the copolymer of L-glutamic acid, L-lysine (GL), tuberculin (PPD) or ovalbumin (OVA)], aged overnight and then trinitrophenylated immediately prior to addition to primed T cells. In each case significant inhibition of the T cell proliferative response was observed when the cultures were performed in the presence of anti-TNP antibody. The inhibition was specific in that macrophages pulsed with the copolymer L-glutamic acid, L-tyrosine (GT, which cannot be readily trinitrophenylated) were not inhibited when treated as above and then cultured in the presence of anti-TNP antibody. Although the mechanism of inhibition by anti-TNP antibody is unknown, it is likely that capping and shedding or endocytosis of antigen occurs, as $F(ab)_2$ but not F(ab) monomers were capable of inhibiting the proliferative response. Surprisingly, under no conditions did we observe inhibition of the response to macrophages which had been freshly pulsed with antigen and immediately TNP-modified prior to addition to culture. The reasons for this finding are not yet clear. It is possible that immediately after pulsing a major portion of the antigen that is taken up is intracellular and thus unavailable to the trinitrophenylation procedure. Alternatively, it is possible that an intracellular processing, handling, or modification step is mandatory to create an immunogen capable of stimulating T cells. This latter step may indeed be the crucial procedure mediated by the products of the Ir genes. We will continue our functional studies in this area in an attempt to determine if antigen must enter the internal milieu of the macrophage and then after a certain period of time be re-expressed on the outer surface of the macrophage. By using antigens such as insulin, we should be able to define if the insulin molecule is altered or split inside the macrophage. For example, as strain 2 guinea pigs recognize determinants in the insulin A chain it will be important to determine if the response to inulin can be blocked by TNP-labeling insulin pulsed macrophages. As the only lysine in the insulin molecule is in the B chain, blockade of the response of strain 2 guinea pigs by anti-TNP would suggest that the insulin molecule is expressed unaltered on the macrophage surface. A major effort will be made to define the biochemical basis of macrophage processing of antigen. Our initial goals are to confirm our functional studies with a direct biochemical demonstration of antigen on macrophage membranes. If these studies are successful, we will attempt to define associations between nominal antigen and Ia antigens and to determine differences in antigen processing between responder and non-responder strains.

II. Role of Ia Antigens in CHO Induced T Cell Activation

We have analyzed in depth the role of Ia antigens in T cell stimulation induced by macrophages whose cell surface has been modified by treatment with sodium periodate or neuraminidase-galactose oxidase to create an active aldehyde (CHO). In this experimental system macrophages are required for T cell activation, but the macrophage need not bear the active aldehyde on its surface as significant T cell activation is observed when the T cell is modified and then cultured with normal unmodified macrophages. We have previously demonstrated that both syngeneic or allogeneic macrophages are equally active in this model and that the proliferative response can be specifically inhibited by anti-Ia sera directed to the macrophage even if it does not bear the active aldehyde. To further investigate the genetic regulation of the response to CHO-macrophages, we have examined whether two distinct populations of F_1 T cells respond to CHO-macrophages from one or the other parental strain. Aldehyde induced stimulation with one parental macrophage appears to specifically activate all populations of F_1 T cells in spite of the fact that the activation can be blocked by anti-Ia sera directed toward the stimulatory macrophage. These results suggest that products of the I-region play a critical role in the induction of CHO induced T cell proliferation, but are capable of mediating this function in a genetically unrestricted fashion. Furthermore, it is possible that the Ia antigens themselves may function as triggers or second signals for T cell proliferation. Our working hypothesis is that the CHO is a stimulus to bring macrophages and T cells into direct physical contact. Following this non-specific physical interaction, the Ia antigens somehow trigger T cell proliferation. Experiments are planned to analyze CHO induced T cell activation on both a morphological level and as a model system for the study of T cell activation at the single cell level using cloned lines of antigen specific T cells and electrophysiological techniques.

III. Production of Monoclonal Antibodies to Guinea Pig Cell Surface Antigens

Our interests over the past few years have shifted from a detailed analysis of the serologic makeup of the MHC of the guinea pig (the GPLA complex) to more functionally oriented studies of the role of the products of the MHC in the regulation of immunocompetent cell interaction. We feel that monoclonal antibodies to both MHC products and other cell surface antigens would be of great aid in the proposed biochemical studies of macrophage T cell interaction.

We have been quite successful in the production of monoclonal mouse antibodies to guinea pig Ia antigens. The availability of an Ia positive B cell leukemia of strain 2 guinea pigs (EN-L_2C) and its Ia-negative variant (BZ-L_2C) have allowed the development of rapid and accurate screening techniques. In these studies BALB/c mice are immunized with EN-L_2C and after boosting, the mouse spleen cells are fused to the NS1 myeloma line. Hybrids secreting antibodies reactive with EN-L_2C, were cloned and ascitic fluid produced. Four independently derived antibodies proved to be specific for Ia antigens. Two of the monoclonals seemed to recognize alloantigenic determinants of strain 2 Ia antigens in that they bound only to strain 2 cells, but not strain 13 cells, and the binding was inhibited by pretreatment of the cells with alloantibodies directed to strain 2 Ia antigens. The other three antibodies were directed to determinants of Ia molecules shared by both strains (framework). All antibodies were cytotoxic in the presence of rabbit complement (titer > 1:100,000) for Ia bearing cells of strain 2, but only one of the two which reacted with strain 13 cells in binding assays was ytotoxic for cells of this strain.

We have compared monoclonal anti-Ia antibodies with alloantibodies to Ia antigens in their ability to inhibit T cell proliferative responses _in vitro_. All four monoclonal antibodies reacted with strain 2 Ia antigens, and all four were capable of inhibiting the strain 13 against strain 2 mixed leukocyte reaction (MLR) by 50-70%; the two monoclonals which reacted with strain 13 Ia antigens were also capable of inhibiting the strain 2 against 13 MLR. In contrast, an analysis of the effects of a single monoclonal antibody on the responses to several antigens demonstrated a selective pattern of inhibition in that the responses to some but not all antigens were inhibited. These results suggest that monoclonal antibodies react with different parts of Ia molecules which may have different functional roles and that certain parts of an Ia molecule participate in the presentation of certain antigens while other regions of the same molecule present different antigens. These results are also consistent with the hypothesis that specific Ir gene function dictates the ability of Ia molecules on macrophages to interact with antigens on macrophages and Ia antigens on macrophages have a limited number of binding sites specific for unique amino acid sequences on antigens. The development of a larger panel of monoclonal anti-Ia antibodies should enable us to evaluate this hypothesis and directly correlate the presence of a given antigenic determinant on an Ia molecule with functional activity in antigen presentation.

We have also succeeded in preparing monoclonal antibodies to guinea pig T cells. One monoclonal antibody GPT1 reacts with an antigen present on

70-80% of peripheral T cells but only present on 10-15% of thymocytes. Treatment of lymph node T cells with GPT1 in the presence of rabbit complement results in a marked reduction of the response of those cells remaining alive to stimulation by alloantigens but has no effect on their response to stimulation by soluble protein antigens or mitogens. A second monoclonal (GPT2) appears to react with a 45,000 dalton molecule present on all peripheral T cells as well as thymocytes. Treatment of peripheral T cells with GPT2 in the presence of complement results in an almost complete elimination of the mitogen, antigen and alloantigen response. We are now developing reagents specific for different populations of macrophages. As only 10-20% of guinea pig macrophages bear Ia antigens and this subpopulation appears to have all the stimulatory activity in both antigen, mitogen and alloantigen presentation, it is crucial to define whether this subpopulation is of the same monocyte-macrophage lineage as the majority of the peritoneal exudate cells. Monoclonal antibodies appear to be the ideal way to approach this question serologically. We are also attempting to produce monoclonal antibodies to epidermal Langerhans cells, a unique population of antigen presenting cells which comprise 3% of epidermal cells.

IV. Induction of Transplantation Tolerance Following Allogeneic Spleen Transplantation

This series of experiments is a collaborative investigation with Dr. H. Bitter-Suermann of the Department of Pathology at Georgetown University Medical School. Dr. Bitter-Suermann developed a model in rats where in certain Ag-B incompatible combinations immunocompetent rat spleen allografts could survive indefinitely in the absence of immunosuppression. Accepted spleen allografts induced a donor specific unresponsiveness for skin allografts which continued to operate even after spleen allograft removal. In order to establish the cellular basis for this specific unresponsiveness we have adapted this model to the guinea pig system. These studies cannot be performed in mice because the blood vessels are too small for the vascular anastomosis. 85% of strain 13 spleens transplanted to strain 2 animals survive for periods longer than three months; in contrast, only 20-30% of strain 2 spleens grafted to strain 13 animals survive. F_1 to parental grafts exhibit about an 80% survival in either direction. Of interest is that we can successfully transplant parental spleens to F_1 animals with minimal sequelaeof graft versus host disease. Spleen allografts 13 to 2 shielded concomitantly inserted strain 13 skin allografts from rejection. Recently, we have shown that strain 2 recipients of strain 13 spleen allografts have a marked (∿ 90%) suppression of the MLR against strain 13 stimulator cells from a period of 2 weeks to 2 months following grafting. We are now in the process of determining the basis for this unresponsiveness as well as its specificity.

Significance for Biomedical Research:

The major histocompatibility complex plays a critical role in the regulation of several aspects of the immune response. Studies in man as well as in several experimental animal systems have demonstrated that genes within the MHC control disease susceptibility. Our studies are designed to determine the mechanism of action and site of expression of MHC-linked immune response genes. Although we have elected to study experimental models where simple protein antigens are used, the results we will obtain should have direct applicability and should give important insights to the analysis of the role of immune response genes in the control of disease susceptibility in man.

PUBLICATIONS:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-AI-00147-05 LI

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)
The Mechanism of Activation of Thymus-Derived Lymphocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
8.1	7.1	1

CHECK APPROPRIATE BOX(ES)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project focuses on the function of immune response (Ir) genes in the activation of thymus-derived (T)-lymphocytes using radiation-induced bone marrow chimeras, a secondary T cell proliferation assay, and a newly developed technique for cloning antigen-specific T lymphocytes. With the chimeras we have been able to generate evidence which suggests that Ir gene products are the serologically detectable Ia antigens which function primarily in macrophages of both the thymus and peripheral tissues and are involved in the presentation of antigen. Using the cloning technique we have demonstrated major histocompatibility complex (MHC) restriction at the level of a single cell and its progenitors proving that allogeneic suppression cannot explain the requirement of T-cells to see antigen in association with self MHC products. Finally, studies of the proliferative response to the antigen, pigeon cytochrome c, demonstrated that two complementing Ir genes could control the response to a single antigenic determinant and that both gene products must be present in the same antigen-presenting cell in order to stimulate a response.

Our previous studies on the localization of immune response genes had demonstrated a requirement for the antigen-presenting cell to come from a high responder strain. In pursuing these studies further, we have now demonstrated that the T-lymphocytes do not have to be from a genotypic high responder provided that the cells mature in the environment of a high responder. This conclusion was reached from studies with radiation-induced bone marrow chimeras. T-cell depleted bone marrow cells from low responder mice were injected into lethally irradiated high responder F_1 mice and allowed to develop into mature T cells. Such chimeras did not respond to the antigen, suggesting the requirement for at least one high responder cell type. Because we knew that the antigen-presenting cell was one such cell type, we had to provide this cell in order to assess the requirements for the T-lymphocyte. This was accomplished by transferring chimeric spleen cells to an acutely irradiated F_1 animal along with T-cell-depleted F_1 bone marrow. These animals now responded to the antigen. Thus, only high responder antigen-presenting cells were required. However, the environment in which the T cells develop was also critical. When chimeras of the type high responder F_1 bone marrow into lethally irradiated low responder hosts were created, they failed to respond to the antigen even though they possessed high responder antigen-presenting cells derived from the bone marrow. In $P_A + P_B \rightarrow (P_A \times P_B)F_1$ chimeras, the chimeric P_A cells remaining after killing with anti-P_B antisera plus complement were able to mount Ir-controlled responses to antigen in association with responder P_B antigen-presenting cells but not nonresponder P_A antigen-presenting cells. This interaction was blocked by antisera directed against the antigen-presenting cell but not by antisera directed against the T cell. Thus, chimeric T cells of nonresponder genotype become phenotypic responders by learning to recognize antigen in association with responder Ia determinants on antigen-presenting cells. These data suggest that Ia antigen-bearing molecules are in fact the Ir gene products.

As an offshoot of these experiments, we also studied the turnover of the antigen-presenting cell after irradiation and bone marrow reconstitution. The turnover of the antigen-presenting cell in the spleen and peritoneal cavity is rapid with no cells capable of presenting antigen detectable on day 4 after irradiation. Such cells are completely replaced by cells of bone marrow donor phenotype by day 7 after reconstitution with 10^7 cells. In contrast, the antigen-presenting cells in the thymus are replaced by donor type cells much more slowly (approximately 2 months). This slow turnover in the thymus might explain why $F_1 \rightarrow P$ chimeras are restricted to host phenotype since only host antigen-presenting cells are around during most of the repopulation of the peripheral T cell pool. We have recently obtained support for this hypothesis, by depleting peripheral T cells of $F_1 \rightarrow P$ chimeras with anti-lymphocyte serum and cortisone and allowing the animals to again regenerate new T cells. This second time the T cells develop in the presence of donor F_1 presenting cells and the peripheral T cells were found to be no longer restricted to host phenotype.

During the past year the laboratory has achieved a major technical break-through with the development of a new method for cloning antigen specific, proliferating T lymphocytes directly from primed murine lymph nodes. The T lymphocytes are stimulated in vitro with antigen for three days and then cloned in soft agar in the presence of antigen. After 5 days the colonies are picked with the aid of a stereomicroscope and the cells expanded in liquid culture using antigen, irradiated spleen cells and a growth factor derived from Concanavalin A stimulated splenocytes. Recloning in the absence of this factor was performed to ensure clonality of the cells. The clonal precursor frequency was low (1 to 2 x 10^{-5}) and showed the expected dependence on Ir gene status i.e. cells from low responder strains yielded fewer colonies than cells from high responder or F_1 strains. All the clones were antigen specific and in particular clones derived from DNP-OVA immunized mice were carrier specific, i.e., they could be stimulated with the ovalbumin (OVA)carrier alone but not with the dinitrophenyl hapten (DNP) on another carrier such as DNP-GLA. However, the clone was capable of detecting the hapten since a 3-fold higher concentration of OVA was required to give an equivalent stimulation to that of DNP-OVA. In addition to their specific antigen, the cells also required that the irradiated spleens be histocompatible at the I-A subregion in order to stimulate T cells to proliferate. The fact that a clone of T cells was I-A restricted in its antigen recognition demonstrated for the first time that suppressor T-cell function cannot account for the phenomenon of major histocompatibility complex (MHC) restriction in cell interactions.

In deciphering the mechanism of action of Ir gene products, it was deemed necessary to investigate the types of antigenic determinants recognized by T lymphocytes to ascertain whether the repertoire of receptors on these cells was any different from that of B lymphocytes. To facilitate such experiments we turned to the small globular proteins as antigens, because they provided us with probes for which we possessed a thorough understanding of the amino acid sequence and a precise knowledge of the three-dimensional structure. Thus, we could localize the antigenic determinants by cross-stimulation with species variants and enzymatic or chemical cleavage fragments of the molecules. For the last few years we have focused on the T-lymphocyte proliferative response of B10.A and B10.S(9R) mice to pigeon cytochrome c. This response appears to be directed at a single determinant comprised of three amino acid residues from different parts of the molecule: the isoleucine at position 3, the glutamine at position 100 and the lysine at position 104. These three residues lie next to each other on the outer surface of the back face of the molecule. Although these results suggest that T cells recognize three dimensional determinants, cyanogen bromide cleavage fragments of the molecule could stimulate the T cells primed to the whole molecule. In fact, one fragment, residues 81-104, containing only two of the three critical amino acids and lacking any of the α helical content this segment possesses in the native structure, stimulated better than the whole molecule on a molar basis. Furthermore, this fragment was immunogenic and the response was under the control of two MHC-linked Ir genes, just as was the response to the whole protein. These results have raised the possibility that T-cell receptors recognize anigen fragments on the surface of macrophages rather than intact molecules. Mixing experiments demonstrated that many of the responding T cells were recognizing the two critical amino acids found in the fragment as part of the same determinant. This result demonstrated for the first time that two Ir genes could control the immune response to a single antigenic determinant. Finally, antigen-presentation experiments showed that both Ir gene products must be expressed in the same presenting cell in order to generate an immune response to pigeon cytochrome *c*.

Significance to Biomedical Research

Our attempts to understand the mechanism of T-lymphocyte activation are of fundamental importance to a basic understanding of how the immune system functions. Since this system plays a critical role in the bodies defense mechanisms against infectious diseases, tumors and transplanted tissues, achieving our goal would hopefully provide major insights into how to manipulate the immune system for the benefit of the patient.

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LABORATORY OF INFECTIOUS DISEASES

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SUMMARY STATEMENT -- Annual Report of the

Laboratory of Infectious Diseases
National Institute of Allergy and Infectious Diseases
October 1, 1979 to September 30, 1980

The main focus of the research programs of LID continues to be the natural history and prevention of the three major uncontrolled, acute infectious diseases of man--acute respiratory tract disease, viral hepatitis, and acute gastroenteritis.

HEPATITIS

Impressive progress has been made during the past year in elucidating the properties of the hepatitis viruses and developing strategies and procedures for prevention of disease caused by these agents.

Hepatitis A Virus (HAV) - Hepatitis A virus (HAV) resembles most closely the picornaviruses: It is a 27 nm particle which exhibits cubic symmetry, contains four polypeptides with molecular weights similar to those of the picornaviruses and has a single stranded linear RNA genome. However, it is more heat resistant than the picornaviruses, exhibits a wider range of buoyant densities and may have a somewhat smaller genome. HAV was recently isolated in tissue culture for the first time by Provost. HAV was successfully recovered in fetal rhesus kidney diploid cell culture only after the virus had been passaged 31 times serially in marmoset monkeys. Still more recently Frosner recovered HAV directly from feces in Alexander hepatoma cells, a transformed human cell line that probably contains the complete hepatitis B virus genome. Independently we also attempted to isolate HAV directly from clinical materials in a cell line suitable for vaccine development. Immunofluorescence and, to a lesser extent, radioimmunoassay (RIA) were employed to detect evidence of virus multiplication. Three strains of HAV were serially passaged in marmosets up to 9 times, and marmoset-passaged virus was compared, at various passage levels, with HAV present in original fecal specimens for capacity to grow in tissue culture. Each of the 3 marmoset passaged strains grew in culture, but the HM 175 strain grew best. For this reason subsequent efforts were limited to the HM 175 strain. The HM 175 strain of HAV was successfully isolated in primary African green monkey kidney (AGMK) cell culture directly from the patients' feces as well as after 6 passages in marmosets. Multiplication of virus at early passage levels *in vitro* was slow, requiring 8 or more weeks to achieve moderate to intense fluorescence in cell culture. Subcultivation of virus was carried out by passage of live cells since all of the detectable antigen was cell-associated. HAV is currently in 7th passage in primary AGMK cells, and the interval required for replication *in vitro* has shortened from approximately 8 weeks to 3-4 weeks. Virus isolated directly from feces is growing as well as marmoset-passaged virus. Because HAV is difficult to grow in tissue culture and because a number of questions remain unanswered concerning its molecular virology and taxonomy, we are currently attempting to clone the HAV genome in *E. coli*. In addition, monoclonal antibodies are currently being prepared in an attempt to characterize the various antigenic determinants of HAV. (Daemer, Ticehurst, Feinstone, McAuliffe)

Hepatitis B Virus (HBV) - Hepatitis B virus (HBV) is a unique, 42nm virus of complex structure that contains a double-stranded circular DNA with a single stranded gap spanning 20-50% of the genome. Recently three other viruses with similar characteristics were discovered: The woodchuck hepatitis virus, (WHV), the Beachey ground squirrel virus and the duck hepatitis virus. Undoubtedly other members of this group will be recognized in other species. HBV is the cause of considerable morbidity and mortality, accounting for 30-50% of clinical hepatitis diagnosed in the U.S. However, it is in Asia and Africa where the virus causes the greatest damage. In these areas 5 to 10% of HBV infections become chronic and a significant proportion of individuals so affected develop chronic hepatitis or hepatic cell carcinoma. For these reasons control of HBV is an important public health goal.

Modifications made in the formulation of the purified inactivated HBs Ag vaccine have yielded a highly immunogenic preparation, capable of stimulating antibody in a majority of recipients receiving as little as 5 ug of vaccine. Furthermore, the antibody response is rapid. Sixty to 80% of vaccinees develop antibody within one month of vaccination and 95% seroconvert by completion of the vaccination series. The NIAID hepatitis B virus vaccine produces antibody in approximately 2-3 times as many recipients by one month after vaccination as other vaccines currently undergoing testing. For this reason we believe that the NIAID vaccine will be useful in those situations where rapid antibody response is essential, such as interruption of the maternal-fetal transmission of HBV that occurs commonly in Asia. Plans are underway to carry out an efficacy trial of the NIAID hepatitis B vaccine in such a setting. (McAuliffe, Johnson, Purcell)

Non-A, Non-B Hepatitis Viruses - As serologic tests for HAV and HBV infection are more widely applied it has become apparent that non-A, non-B hepatitis agents are widely distributed and are associated with a significant proportion of clinical hepatitis cases, perhaps 25% of all clinical cases and as much as 90% of post-transfusion hepatitis. The epidemiology of the non-A, non-B agents resembles that of HBV quite closely: the agents are primarily transmitted by blood and blood products and lead to chronic infection in a high proportion of patients. A number of such agents have been successfully transmitted to chimpanzees, but despite intensive efforts, an antigen-antibody system unequivocally specific for non-A, non-B infections has not been detected. An exception is delta antigen, an antigen associated with a unique non-A, non-B agent.

Transmission of non-A, non-B agents to primates was extended during the past year. For example, two strains were titrated for infectivity in chimpanzees. One strain that produced cytoplasmic changes in liver cells had an infectivity titer of approximately 10 chimpanzee doses (CID) per ml, while another strain that produced predominately nuclear changes had a CID of 10^7-10^8 per ml. These titered suspensions will be used to characterize the two non-A, non-B viruses and determine their relationship to each other. The two types of non-A, non-B viruses (i.e., predominately cytoplasmic or nuclear pathology) were successfully transmitted to marmosets. The nuclear changes were reproduced in marmosets but the cytoplasmic changes have not been seen to date.

In collaborative studies with Dr. Khorshed Pavri, we found that two outbreaks of epidemic water-borne hepatitis and one cluster of cases of endemic hepatitis in India could not be serologically related to infection with HAV or HBV. Thus, these cases, which resembled type A hepatitis in many respects, appeared to be caused by one or more non-A, non-B agents. It is not clear whether these agents represent one or more previously unrecognized serotypes of HAV or whether they are an entirely new group of agents. Attempts to transmit them to chimpanzees and marmosets are currently in progress. (Feinstone, Johnson, Purcell)

Delta Antigen- In 1977, Mario Rizzetto discovered a new antigen in patients with chronic type B hepatitis. This antigen, detected by fluorescence, was found in hepatocyte nuclei of patients with chronic type B hepatitis and was distinct from hepatitis B core antigen. Recently, delta agent was characterized further by Dr. Rizzetto during his tenure as a Visiting Scientist in LID. In collaborative studies with Dr. Gerin and his staff, delta antigen was found to be associated with a transmissible agent that required growth of HBV for its synthesis which in turn suppressed the synthesis of its HBV helper. This suggested that the delta antigen was a defective interfering agent. Delta antigen could be detected by immunoflourescence in the liver of experimentally infected chimpanzees but could not be detected in the serum until centrifugal pellets of serum were treated with detergent. This suggested that delta antigen was enclosed within a particle. When samples of chimpanzee plasma containing delta antigen were subjected to centrifugal separation, delta antigen copurified with 35-37nm particles that morphologically resembled large HBsAg particles. Furthermore, delta particles coaggregated with 22nm HBsAg particles when a mixture of these particles was exposed to antibodies directed against HBs Ag. Thus, delta was an internal component of a particle incapsidated by HBsAg. When such particles were extracted for nucleic acid, a low molecular weight (approx. 500,000 daltons) RNA was detected. This RNA also appeared to be enclosed, with delta antigen, inside a coat of HBsAg. Delta antigen was readily transmitted to chimpanzees either acutely or chronically infected with HBV, but could not be transmitted to HBV-immune chimpanzees. Electron micrographic changes similar to those seen during acute non-A, non-B hepatitis infection were detected in such chimpanzees, suggesting that delta antigen may share some characteristics with other non-A, non-B agents.

Dr. Rizzetto developed a radioimmunoassay for delta and anti-delta and performed seroepidemiologic studies. He found that delta antigen had a worldwide distribution and was highly endemic in Italy, especially in the southern region, where it was assocaited with 50% of acute HBV infections. In Europe and the U.S. evidence of infection with delta antigen was found most frequently in those individuals extensively exposed to blood and blood products (hemophiliacs, illicit drug users, etc.). In summary, delta antigen appears to be a unique transmissible agent with characteristics unlike those of any previously described self-replicating agent. (Purcell)

ACUTE GASTROENTERITIS

Background - Important advances have been made in elucidating the etiological agents of acute infectious, nonbacterial gastroenteritis, a syndrome that affects a broad segment of the population. It appears that viral gastroenteritis consists of at least two entities with distinct epidemiological characteristics. One, designated epidemic viral gastroenteritis, tends to occur in family or community-wide outbreaks affecting adults, school-aged children, family contacts and probably young children as well. The illness is usually self-limited and characteristically lasts 24-48 hours. Previously, we demonstrated by immune electron microscopy (IEM) that a 27nm particle -- the Norwalk agent -- was associated with an outbreak of this form of gastroenteritis that occurred in Norwalk, Ohio. In addition, in further studies by our laboratory, particles that resemble the Norwalk agent morphologically have also been associated by IEM with 2 family outbreaks of gastroenteritis, one in Hawaii and the other in Montgomery County (MC), Md. The Norwalk and Hawaii agents appear to be distinct, whereas the Norwalk and MC agents appear to be related. An agent designated Ditchling or "W" has been associated with this form of gastroenteritis in studies in England. In addition, other "small round viruses", some with calcivirus-like morphology, have been associated with outbreaks of gastroenteritis in various parts of the world.

The other form of gastroenteritis, designated sporadic infantile gastroenteritis, represents a severe form of diarrhea affecting infants and young children, that often necessitates hospitalization and parenteral fluid therapy. It is evident from studies in many parts of the world that the human rotavirus is the major etiological agent of serious sporadic infantile gastroenteritis.

Cultivation of Type 2 Human Rotavirus - In last year's annual report we described the successful *in vitro* cultivation of the "Wa" strain of type 2 human rotavirus. Our ability to propagate type 2 human rotavirus in culture made it possible for us to characterize this fastidious agent in greater detail than heretofore possible and to study its antigenic properties as they relate to homotypic as well as heterotypic immunity. An efficient plaque assay was developed for quantitating virus and antibody and for isolating genetically homogeneous populations of virus. Using a plaque reduction antibody system, we confirmed the results of previous immunofluorescence studies that showed the "Wa" virus to be antigenically distinct from bovine, porcine, and simian rotaviruses. In addition, optimal conditions were defined for growing the type 2 human rotavirus in a cell substrate suitable for propagation of virus to be administered to man. Plaque purified virus is currently being grown in African green monkey kidney (AGMK) cell culture and will be evaluated for its virulence in susceptible adult volunteers with low or undetectable intestinal secretory IgA rotavirus antibody. If the culture adapted type 2 human rotavirus proves to be attenuated in this situation it will be tested in successively younger individuals for its suitability as an attenuated vaccine strain. (Wyatt, Kalica, Kapikian, Chanock)

Temperature-sensitive (ts) Mutants of Animal Rotaviruses - As part of our effort to investigate and modify the virulence of rotaviruses, 10 ts mutants of the UK strain of calf rotavirus were isolated after mutagenesis with 5 azacytidine and for 5 fluorouracil. Each of the mutants had a shutoff temperature for plaque formation of 38°C or 39°C and each was relatively stable genetically. Analysis of the ten mutants showed that they belonged to four separate, non-overlapping recombination groups. Polyacrylamide gel analysis of the RNA of these mutants indicated that each exhibited the same pattern as the ts^{+} wild type UK parent. In addition, we recently isolated 6 ts mutants of the SA-11 simian rotavirus which fell into 3 non-overlapping recombination groups. (Greenberg)

Use of ts Mutants to Rescue Non-Cultivatable Human Rotavirus by Gene Reassortment - Except for the "Wa" strain of type 2 human rotavirus, human rotaviruses have not been successfully propagated serially in vitro to high titer. In addition, most animal and avian rotaviruses are extremely fastitious and cannot be cultivated in vitro by current cell culture techniques. For these reasons we developed a strategy for converting fastidious rotaviruses into viruses capable of efficient growth in tissue culture. This was achieved by genetic reassortment during mixed infection with a cultivatable rotavirus. In this manner, the genes of the fastidious rotavirus which restricted growth in vitro were replaced by the corresponding genes from a tissue culture adapted rotavirus. In the first experiments ts mutants of the tissue culture adapted UK or SA-11 virus were used to rescue type 1 or 2 non-cultivatable human rotavirus. Briefly, a non-cultivatable strain of human rotavirus which had been amplified by passage in gnotobiotic calves or which was present in high titer in human stool (Wa) was centrifuged at low speed onto AGMK cells. The cells were then superinfected with a ts mutant of a cultivatable bovine or simian rotavirus (UK or SA11) and the infection was allowed to proceed for 36 hours at 34°C. The infected cell culture harvest was then plated out at restrictive temperature with or without UK or SA11 antiserum. Using this technique, we recovered reassortant viruses that grew to high titer and were neutralized specifically by hyperimmune guinea pig type 1 or type 2 human rotavirus antiserum. Preliminary RNA analysis of these clones disclosed that they were indeed reassortant viruses. It was of interest that the neutralization and serotype specificities appeared to reassort independently suggesting that the capsid protein reacting with neutralizing antibodies differs from that measured in the immune adherence hemagglutination serotyping reaction. Gene rescue should prove useful in propagating other non-cultivatable human rotaviruses as well as fastidious animal rotaviruses such as epidemic disease of infant mice (EDIM). Appropriate analysis of such reassortants should allow us to construct a map of the rotavirus genome and to identify the location and function of each of its gene products. (Greenberg, Wyatt, Kalica, Kapikian, Chanock)

Cultivation of Reassortant Rotaviruses With Type 1 Human Specificity - As described in last year's report a cultivatable mutant of type 2 virus emerged during serial passage of the virus in gnotobiotic piglets. The procedure previously used for in vitro cultivation of the type 2 human rotavirus, however, was not successful for type 1 virus. Type 1 virus could not be passaged serially in piglets and thus another strategy for growing the virus in tissue culture was needed.

For this reason we attempted to rescue non-cultivatable type 1 human virus by co-infection with a cultivatable bovine rotavirus ts mutant. Selection of the desired reassortant with type 1 human specificity was achieved by exposing progeny from the mixed infection to high titered, specific bovine rotavirus antiserum and then plaquing surviving virus at restrictive temperature to eliminate ts virus. Using this procedure reassortants were recovered that grew to high titer in tissue culture, produced plaques with high efficiency and were specifically neutralized by hyperimmune type 1 human rotavirus antiserum. It should be emphasized that this approach provides us with a mechanism for generating cultivatable virus with type 1 human rotavirus outer capsid protein that is recognized by specific neutralizing antibody. Such reassortants should prove useful for immunoprophylaxis of type 1 rotavirus disease in man. Finally, it should be noted that analysis of type 1 human rotavirus reassortants that grow well in cell culture should allow us to identify the genes that restrict *in vitro* propagation of naturally occurring type 1 viruses. (Greenberg, Wyatt, Kalica, Kapikian, Chanock)

Biophysical, Biochemical and Immunologic Studies of Rotaviruses - Procedures for study of viral RNA by gel electrophoresis were refined to enable us to differentiate the corresponding genes of different viruses by their mobility in gel. In this manner reassortants can be genotyped and the parental origin of each of their genes ascertained. This type of analysis will provide the basic infrastructure for gene coding assignments and the genetic study of virulence.

Single stranded (ss)RNA transcripts of rotavirus genomic RNA are now under study. These ssRNA transcripts are produced within viral cores by a virion RNA polymerase. Such transcripts should prove helpful for genotype analysis by nucleic acid hybridization and for coding assignment of rotavirus genes by translating ssRNAs in a cell-free system. (Kalica)

Hybridomas are being developed for use in characterization of individual gene products and for analysis of variation among corresponding genes from rotaviruses derived from different host species. (Greenberg, Wyatt)

Norwalk Viruses of Acute Gastroenteritis - The development of a radioimmunoassay (RIA) for detection of Norwalk antigen and a radioimmunoassay blocking (RIABL) test for detection of antibody to the Norwalk agent were described in a previous annual report. The availability of the RIA and RIABL has permitted us to continue and expand epidemiological studies of Norwalk infection. Our survey of outbreaks of gastroenteritis was extended during the past year. To date approximately 75 separate outbreaks have been studied serologically for Norwalk infection and approximately one third were associated with the Norwalk virus. The latter group includes several large, well-documented waterborne outbreaks and 2 large oyster associated outbreaks (one in Australia and one in Florida).

Recently after considerable difficulty, we succeeded in purifying the Norwalk virion. A soluble viral protein found in the stools of infected individuals was also purified. The specificity of the proteins released from dissociated, iodinated, purified virions was established by immunoprecipitation using paired sera from volunteers infected with Norwalk virus. The major structural protein of the Norwalk virus had a molecular weight of approximately 59,000 daltons. This finding suggests that the Norwalk virus is most likely a calicivirus, rather than a parvovirus as thought previously. The soluble viral protein, which is the major antigenic form found in stool, had a molecular weight of approximately 30,000 daltons. The relationship of the soluble protein to the larger virion protein is unclear, however, both are specifically immunoprecipitated by human antibodies that develop during convalescent from Norwalk virus illness. (Greenberg)

INFLUENZA VIRUS GENETICS AND BIOLOGY

Characterization of ts-1A2 Recombinant Viruses - The A/Udorn/72 *ts*-1A2 virus with *ts* mutation(s) on the P3 and P1 genes has been evaluated as a donor of these *ts* genes to new variants of human influenza A virus. The work this year *involved* the genotyping of fifteen A/Alaska/77-*ts*-1A2 (H3N2) and eleven A/Hong Kong/123/77-*ts*-1A2 recombinants. Genotype of recombinant viruses was determined by measuring the migration rate of virion RNA from parental and recombinant viruses on polyacrylamide gel or by antigenic analysis using conventional antiserum or monoclonal antibody. The genotyping of the *ts*-1A2 recombinants confirmed our assignment of the locus of the *ts* genes that had been established previously by complementation-recombination analysis. More important, however, was the demonstration that the two *ts*-1A2 *ts* genes were the determinants of (a) growth restriction of *ts*-1A2 recombinants in animals and (b) attenuation of these viruses for man. In one instance, only the *ts* genes of the *ts*-1A2 donor were transferred into a recombinant bearing H1N1 surface antigens. This recombinant was satisfactorily attenuated for doubly seronegative (susceptible) individuals indicating that the two *ts*-1A2 *ts* genes (P1 and P3) can act as the sole determinants of attenuation. Thus, the two *ts* genes are capable of producing the desired level of attenuation of a wild type virus without the cooperation of other genes from the *ts*-1A2 donor virus. The observation that three influenza A viruses belonging to two subtypes could be satisfactorily attenuated by the transfer of the two *ts*-1A2 *ts* lesions supported the hypothesis underlying our approach to the production of live influenza A virus vaccines, namely wild type virus can be attenuated to a specific desired level by the introduction of genes bearing defined *ts* mutations. (Murphy, Massicot)

Locus of the mutation that suppressed the ts phenotype of a ts-1A2 recombinant- A seronegative child was administered an A/Alaska/77-*ts*-1A2 recombinant that had a 37°C shutoff temperature for plaque formation. This child shed virus on days 7, 8, and 9 that produced plaques efficiently at 39°C. However, this altered virus failed to produce plaques at 40°C, a temperature at which wild type A/Alaska/77 virus produced plaques efficiently. The A/Alaska/77 *ts*$^{+}$ isolate was shown by segregation analysis to contain a *ts* P3 gene and a mutation on another gene that suppressed the *ts* phenotype of this *ts* P3 gene. The work this year centered on identifying the locus of the suppressor mutation. Seventeen *ts* segregants derived by mating the *ts*$^+$ isolate with a wild type virus were genotyped to determine which gene in each *ts* segregant was always derived from wild type virus. The P2 gene in each of the *ts* segregants was derived from wild type virus. In contrast, the P3 gene came from the *ts*-1A2 *ts*$^+$ isolate. At the other gene loci there was independent segregation indicating that none of these genes bore the suppressor mutation. In this manner, the locus of the suppressor mutation was identified as the P2 gene. This finding suggests that the P2 and P3 gene products interact physically because the altered P2 gene product can suppress the temperature sensitivity of the P3 gene product. Previously, the P3 and P2 genes were shown to be involved in c-RNA and v-RNA synthesis respectively, and were not thought to interact. The data from the present study suggests that they do indeed interact. (Tolpin, Murphy)

Restoration of Virulence of ts-1A2 Recombinant - The clinical significance of the new mutations that developed in the *ts*-1A2 recombinant was assessed to determine if there was a restoration of virulence associated with those genetic changes. The *ts*$^+$ virus recovered on day 9 was cloned at permissive temperature (34°C) and then restrictive (39°C) temperature. Subsequently the *ts*$^+$ virus was administered to seronegative adult volunteers at a dose of $10^{4.0}$ or $10^{5.0}$ TCID$_{50}$. The quantity of virus shed, the pattern of virus shedding and the illnesses observed were comparable to those seen in volunteers infected with wild type virus. These results suggest that the *ts* phenotype of the *ts*-1A2 recombinant is a major determinant of attenuation. The high titer of *ts*$^+$ virus shed by the original vaccinee (10^5 TCID$_{50}$ per ml of nasal wash fluid) suggests that restoration of the *ts*$^+$ phenotype might lead to transmission of altered virus to suceptible contacts under the appropriate conditions of exposure. Genetic instability leading to restoration of the *ts*$^+$ phenotype and the possibility of spread of *ts*$^+$ virus to contacts precludes widespread use of *ts*-1A2 recombinants for immunoprophylaxis despite the fact that these recombinants are satisfactorily attenuated for doubly seronegative (highly susceptible) individuals. (Tolpin, Murphy)

Evaluation of Cold-Adapted Recombinants in Adults - Studies were carried out this year with three cloned A/Alaska/77 (H3N2) *ca* recombinants. One recombinant possessed all six transferable A/AA/6/60 genes (i.e., genes other than those coding for the surface glycoprotein hemagglutinin and neuraminidase antigens) and the other two received only 5 of the 6 transferable genes with a wild-type gene at the M or NS locus. Initially, the *ca* recombinant bearing all six transferable A/AA/6/60 genes ("6" *ca* gene recombinant) was found to be satisfactorily attenuated and antigenic in seronegative adult volunteers. However, only 18 of 24 volunteers were infected. Next, the *ca* recombinants bearing only five of the six transferable genes were evaluated. These "5" *ca* gene recombinants were identical to the recombinant bearing all six transferable genes except that they had a wild type gene substituted at the NS or M locus. If the M or NS gene of the *ca* donor strain is a major determinant of attenuation, loss of such an attenuating gene should be associated with increased virulence. Virulence of the "5" *ca* gene recombinants was assessed by determining the duration and magnitude of virus shedding and the clinical response of seronegative adult volunteers to these viruses. The "5" *ca* gene

A/Alaska/77 recombinants were each satisfactorily attenuated and were indistinguishable from the "6" gene A/Alaska/77 recombinant as regards virulence. Each of the recombinants was shed in significantly lower quantity and for a shorter duration than was wild type A/Alaska/77 virus. These observations suggest that the M and NS genes are not significant determinants of attenution of *ca* recombinant viruses. Studies performed previously suggested that the RNA-2 gene of the A/AA/6/60 *ca* virus was not a major attenuating gene. Considered together,these results suggest that RNA-1, RNA-3 and the NP genes, which are present in all *ca* recombinants that have the *ts* and *ca* properties, are most likely the major attenuating genes of the A/Ann Arbor/6/60 *ca* donor virus. However, one cannot rule out the possibility that one or more wild type genes present in a *ca* recombinant undergo mutation during the recombination and cloning manipulations which are performed at 25°C, a temperature undoubtedly highly selective for spontaneous mutation.

One *ca* recombinant, the A/Alaska/6/77 CR29 clone 2, which received all six non-surface antigen genes from the *ca* parent, had a 39°C shutoff temperature but was attenuated in seronegative adults and children. Chemically mutagenized *ts* viruses with a 39°C shutoff temperature that were studied previously retained virulence for seronegative adults. The present findings suggest that mutations other than *ts* mutations play a major role in attenuation of *ca* A/AA/6/60 recombinant viruses for man. (Murphy)

STUDY OF INFLUENZA VIRAL GENE REGULATION AND EXPRESSION THROUGH USE OF RECOMBINANT DNA TECHNIQUES

Notable advances were made during the first complete year of our effort to elucidate regulation and expression of influenza viral genes through the use of recombinant DNA techniques. Our general plan for this approach includes two phases. The first phase involves cloning each of the eight influenza gene segments using the *E. coli* K12-plasmid system (approved MUA #91). The second phase involves the use of purified clones of influenza DNA sequences to examine viral gene expression in animal cell culture and to produce vRNA in eukaryotic cells. To carry out this second phase we propose to utilize defective SV40 (lacking the late region of the geonme that codes for capsid proteins) for construction of influenza-SV40 hybrid DNA molecules (approved MUA #110). Introduction of the appropriate influenza-SV40 hybrid DNA molecules into eukaryotic cells may lead to transcription or translation of influenza viral DNA depending upon its orientation of insertion. Our goal is to devise procedures which will permit conversion of influenza DNA back to an influenza RNA negative strand and eventually transfer such RNA into an influenza virus. In this manner, stable site-specific mutations, such as deletions, induced in the cloned DNA might be transferred back to the influenza virus. Thus, it may be possible to develop stable mutants for experimental study and for use in immunoprophylaxis.

Initially, positive and negative cDNA strands were prepared separately by reverse-transcription. The positive strands of cDNA were transcribed from genomic RNA segments by using a specific dodecamer DNA primer with a sequence complementary to the conserved 3'-terminal nucleotides of virion

RNA. The negative strands of cDNA were transcribed from cytoplasmic viral mRNA segments by using an oligo(dT) primer. The single stranded DNA molecules from both preparations were annealed to generate double-stranded DNA segments. The in vitro synthesized influenza DNA segments were inserved into plasmid pBR322 at the specific Pst I site using the dG-dC tailing technique. The hybrid DNA molecules were used to transform recipient E. coli and transformants containing influenza gene sequences were identified by hybridization. In this manner, we obtained putative full-length influenza virus DNA segments corresponding to genes coding for the nonstructural proteins (gene 8), the matrix protein (gene 7), the hemagglutinin (gene 4) and the neuraminidase (gene 6). This approach should be generally applicable to other influenza virus genes as well as to other negative strand RNA viruses.

The influenza virus specific DNA sequences were characterized by restriction enzyme digestion. Restriction enzyme cleavage sites were positioned on the linear DNA map of each DNA segment and oriented with respect to the 3'-terminus of the virion RNA. Generally, orientation was carried out by forming duplex DNA molecules between the 5'-labelled plus cDNA strand and an excess of the cloned DNA segment and cleaving the duplexes with a restriction enzyme. The labelled terminal fragment which positioned the 3'-end of virion RNA was identified by gel electrophoresis. Using this approach we established the restriction enzyme cleavage maps of cloned DNA sequences corresponding to 4 genes of influenza virus strain A/Udorn/72 (H3N2).

RNA segment 8 of the influenza virus genome is unique in coding for two different polypeptides, NS1 and NS2. These polypeptides are synthesized from separate mRNA species. Using the cloned DNA derived from virion RNA segment 8 (NS DNA) the template locations for the two mRNA transcripts were mapped by the S1 nuclease method. The body of the NS1 mRNA maps at 0.05-0.95 units of the cloned NS DNA and the body of the NS2 mRNA maps at 0.59-0.95 units, suggesting that the two mRNA's share the same poly (A) addition site at their 3' ends. The template locations of the mRNA's on the viral genome segment were confirmed by hybrid-arrested translation experiments in collaboration with Dr. Robert Lamb, Rockefeller University, using fragments of the cloned NS DNA to inhibit the synthesis in vitro of NS1 and NS2 polypeptides. In addition, in these translation experiments certain DNA fragments brought about premature termination of the NS1 polypeptide allowing us to estimate that the termination of translation of NS1 is at 0.76 map unit. Thus, the coding regions of the two mRNA's overlap. Peptide mapping experiments indicated that NS1 and NS2 do not share methionine- or leucine-containing tryptic peptides suggesting that translation of the NS2 mRNA occurs in a reading frame different from that used for NS1. Experiments using primer extension were performed in collaboration with Dr. Lamb to determine more precisely the nucleotide sequences at the 5'-terminus of the NS2 mRNA. From nucleotide sequence determinations performed by Dr. Lamb it was found that the NS2 mRNA contains an interrupted sequence of 473 nucleotides. The nucleotide sequences at the junctions of the interrupted segment are similar to those of the consensus sequences at the splicing sites of intervening regions in eukaryotic mRNA's. In contrast, the NS1 mRNA appears to be continuous and therefore is a linear transcript of the gene segment. The first 56 virus-specific nucleotides at the 5'-end of the NS2 mRNA are the same as those found at the 5' end of the NS1 mRNA. This leader sequence of the NS2 mRNA contains the initiation codon for protein synthesis and codes for 9 animo acids which are common to NS1 and NS2 popypeptides. Both mRNA's contain heterogeneous non-viral oligonucleotides at their 5'-ends (see below).

Complete influenza viral DNA sequences corresponding to each of the eight genes should be useful for further biologic experiments directed toward elucidation of regulation and expression of viral genes. Sequence analysis of the terminal nucleotides of the cloned viral DNA segments indicated that both termini of vRNA were fully represented in clone pFV88 (gene 4), pFV101 (gene 6) and pFV26 (gene 8) indicating that these cloned DNAs contained the complete information present in the viral RNA segments coding for the hemagglutinin, the neuraminidase and the non-structural proteins, respectively. These observations suggest that our procedures for cloning influenza virus gene segments yielded complete copies of viral DNA which should have all the control signals for viral gene replication and expression in eukaryotic cells and thus allow us to convert influenza DNA back into its original viral RNA form.

As just described, our technique of cloning DNA duplexes formed between cDNA strands separately synthesized from virion RNA and cytoplasmic viral mRNA from infected cells yielded complete DNA sequences for both influenza viral RNA and its corresponding mRNA. Such complete cloned DNA was suitable for analysis of nucleotide sequences present at the 5'-terminus of individual cytoplasmic mRNA species. Sequence determination of terminal nucleotides from five independently isolated viral DNA segments showed that additional oligonucleotides were covalently linked to the 5'-terminus of viral mRNA transcripts. The sequences of these additional nucleotides varied among DNA clones of the same gene and of different genes as well. These inserts also varied in length. The heterogeneity of these sequences suggests that they were derived originally from cellular RNA molecules. These findings provide evidence that cellular RNA sequences are utilized to promote influenza viral mRNA transcription in infected cells. (Lai, Markoff, Sveda)

RESPIRATORY SYNCYTIAL VIRUS

Background - RS virus is the major etiologic agent of bronchiolitis and pneumonia of early life, and the need for effective immunoprophylaxis has been clearly established in studies performed throughout the world. This virus has a non-segmented genome and does not undergo genetic reassortment. Initially a series of 7 ts mutants of RS virus were produced by chemical mutagenesis with the expectation that their temperature sensitivity would restrict growth in the target organ, the lungs. Additional cold-adapted strains were derived as possible vaccine candidates.

Evaluation of Mutants in Primates - Two mutants were evaluated in seronegative chimpanzees. A cold-adapted (ca) mutant recovered and passaged only in human diploid cells was provided by the Sandoz

Forschungsinstitut. This virus given to 2 chimpanzees by the intranasal (IN) route induced rhinorrhea which could not be distinguished from that produced by wild type, virulent virus. The ts-1 NG-16 mutant, also grown in human diploid cell culture, was given to 2 chimpanzees which developed rhinorrhea similar in duration and severity to that seen during infection with wild type virus. Both mutants appeared to retain some virulence for the upper respiratory tract suggesting that they were not sufficiently attenuated for use in susceptible infants and young children. On the other hand it may not be possible to reduce virulence below the level exhibited by these mutants and still retain sufficient infectivity to provide effective immunization. Experience with the ts-2 mutant during the past year highlights this dilemma. The ts-2 mutant does not induce rhinorrhea in seronegative chimpanzees, however, it is markedly restricted in infectivity for both seronegative chimpanzees and young children. The latter characteristic severely limits its usefulness as a candidate vaccine strain. (Suffin, Chanock)

Immunosuppressive effect of serum antibody on response of cotton rats to live RS virus administered intramuscularly (IM) - Since an RS virus vaccine is most urgently needed during the first few months of life, the issue of immunosuppression by maternally derived serum antibody represents a potential obstacle to successful immunization in this age group. Work reported last year showed that cotton rats born to mothers which had previously been infected with RS virus were refractory to intramuscular (IM) vaccination with live wild-type virus. In contrast, animals born to mothers without prior exposure to RS virus were successfully vaccinated by the IM route. Although specific immune factors were responsible for the immunosuppressive effect, the nature of those factors, and the amount necessary to block immunization were not determined. We examined the phenomenon of immunosuppression in greater detail by administering pooled cotton rat RS virus antisera intraperitoneally (IP) to seronegative animals which were than immunized IM with live wild-type virus. Those animals which were given antisera were unable to respond to the IM vaccine, whereas the animals which received pooled control sera (free of RS virus antibody) prior to vaccination were successfully immunized. This demonstrated that serum antibody or a concomitant serum immune factor, could produce immunosuppression.

A second experiment, designed to determine the dilution endpoint of serum blocking factors required for immunosuppression, showed that pooled antisera diluted 1:256 was sufficient to completely block IM vaccination, although this amount of antisera did not yield detectable neutralizing antibody in the serum of recipient rats. This suggests that a live virus vaccine administered IM may not be useful in individuals most in need of immunoprophylaxis, since the greatest impact of RS virus occurs during the first six months of life when most infants possess maternally derived, serum neutralizing antibody for RS virus.

The immunosuppressive effect of antibody on parenteral RS virus vaccination raises the possibility of a similar effect during immunization with a live attenuated virus administered into the respiratory tract. To study this possibility, we inoculated seronegative cotton rats IP with the pooled antisera described previously, then administered live, attenuated RS virus (*ts*-1 NG-16) intranasally (IN) the following day. Following challenge with wild type virus (three weeks later) all the animals were shown to be resistant to challenge (IN) with wild type virus, whether they had received immune or control (antibody-free) sera prior to vaccination. It would appear that the immunosuppressive effect seen in IM vaccination is *not* seen during IN vaccination, and that topically applied RS virus vaccines may confer effective immunity independent of antibody in serum at the time of vaccination. (Prince)

MYCOPLASMA

Efforts to assess the significance of pathogenic spiroplasmas centered around the biological and serological characterization of organisms within this group, on development of appropriate models to explore pathogenicity and host response, and on evaluation of various techniques to determine the possible role of these agents in human disease. The recovery of new spiroplasmas from *Ixodes* ticks provides an important lead to the possible role of these organisms in human infections, since these insects are frequently associated with man. Our comprehensive serological analysis of spiroplasmas has shown at least six distinct serological groups. A proposed classification scheme, based upon the serological findings and genetic distinctions (including lack of DNA hybridization) provides an important base to the seroepidemiological study of spiroplasma antibodies in man and vertebrates. The pathological studies performed on spiroplasma strains virulent for vertebrates has also offered some significant insights into host response to these organisms. The absence of a clear inflammatory response in the brain of rats exhibiting central nervous system disease, even in the presence of rather large numbers of spiroplasmas in brain tissue, emphasizes the value of establishing a mechanism to specifically identify the organism in host tissue. Some recent findings on *Mycoplasma pneumoniae* infections in man suggested an unusual carrier state in this disease, the organism being isolated in the absence of any clear evidence of respiratory disease or a specific serological response. (Tully)

HONORS AND AWARDS

Robert M. Chanock

Director, International Reference Laboratory for Respiratory Viruses Other than Influenza, World Health Organization, 1973 - present
Director, International Reference Laboratory for Mycoplasmas, World Health Organization, 1973 - present
Associate Editor, American Journal of Epidemiology
Member, Advisory Board, Archives of Virology
Chairman, Section of Medical Microbiology and Immunology, National Academy of Sciences, 1976-80
Invited speaker, Symposium on Prevention of Major Infectious Diseases: Current Concerns and Future Promise, Univ. of Rochester School of Medicine, New York, September 20-21, 1979
Member, Nominating Committee, National Academy of Sciences, 1979-80
Member, Class Membership Committee, National Academy of Sciences, 1979
Elected Foreign Member, Royal Danish Academy of Sciences and Letters, 1980
Reappointed member, World Health Organization Expert Advisory Panel on Virus Diseases, September 1980-85

Robert H. Purcell

Recipient, Eppinger Prize and invited Participant, 5th International FALK Symposium on Virus and Liver, Basel, Switzerland. Sept. 29 - Oct. 9, 1979.
Invited Speaker, The Eleventh Symposium on Perspectives in Virology. New York City, New York. February 3-5, 1980.
Expert Consultant in hepatitis, U.S.-China Bilateral Science Agreement. Beijing and Shanghai, China. February 28 - March 10, 1980.
Participant, First U.S.-Japan Symposium on Viral Hepatitis. New York City, New York. March 24-25, 1980
Visiting Professor and Noble Wiley Jones Lecturer. University of Oregon Medical School. Portland, Oregon. April 28-30, 1980.
Invited Speaker, 1980 Medical Alumni Weekend Program, Tufts University, Boston, Massachusetts. May 1-2, 1980.
Invited Faculty Member, Digestive Diseases Update, American Gastroenterology Association annual meeting, Salt Lake City, Utah. May 18, 1980.
Howard B. Tickton Memorial Lecturer, George Washington University Medical School. Washington, D.C. May 24, 1980.
Invited Speaker and Workshop Chairman, International Symposium on Non-A, Non-B Hepatitis. Vienna, Austria. June 16-18, 1980.

Albert Z. Kapikian

Invited to attend and to make 2 presentations at the Health Year, 1979 - Congress on Virology and Class 4 Agents, Sept. 17-19, 1979 in Johannesburg, South Africa. The official opening of the new class 4 containment laboratory of the National Institute for Virology took place at the end of the Congress on Sept. 19, 1979
Invited lecturer on Viral Gastroenteritis, Tropical Medical Course, Walter Reed Army Institute of Research, Washington, D.C. July 25, 1980

Albert Z. Kapikian (continued)

Co-chairman of session on Viral Gastroenteritis (Oct. 3, 1979) at Thirteenth Joint Conference on Viral Diseases of The United States - Japan Cooperative Medical Science Program, Oct. 1-3, 1979 in Atlanta, Georgia. Also, co-moderator of roundtable on Viral Gastroenteritis at same conference on Oct. 3, 1979.
Invited (along with Dr. Flewett of Birmingham, England) as temporary adviser to World Health Organization "to provide technical services related to drafting a strategic plan for research in viral diarrhea," March 5,6,7, 1980 at WHO, Geneva, Switzerland.

Joseph G. Tully

Past-Chairman and Member, Board of Directors, International Organization for Mycoplasmology (IOM).
Past-Chairman and Member of Board, IOM International Research Program on Comparative Mycoplasmology (formerly WHO/FAO Program).
Chairman, Subcommittee on Taxonomy of Mycoplasmas, American Society for Microbiology.
Co-Director, Workshop on Detection of Mycoplasmas, Cell Science Center, Lake Placid, N.Y., August 11-14, 1980.
Member, International Subcommittee on the Taxonomy of Mollicutes.
Member, Advisory Committee, Bergey's Manual of Determinative Bacteriology, (9th edition).
Invited speaker, U.S. Dept. of Agriculture Basic Sciences Lecture, Oct. 5, 1979.
Invited speaker, Dept. of Plant Pathology, University of California, Riverside, CA, February 25-28, 1980.
Invited speaker, Workshop on Murine Mycoplasma Infections, Animal Resources Branch, DRR, NIH, March 12, 1980.
Invited speaker, Division of Neuropathology, University of Maryland School of Medicine, May 21, 1980.
Invited contributor, Conference on Current Status of Caprine Pleuropneumonia in US, U.S. Dept. of Agriculture, Hyattsville, MD, May 28, 1980.
Invited speaker, Symposium on Mycoplasma-Host Relationships, 3rd International Conference, International Organization for Mycoplasmology, Custer, So. Dakota, Sept. 3, 1980.
Invited contributor, Symposium on Bacterial Vaccines, NIH, Sept. 15-18, 1980.

Brian R. Murphy

Invited speaker, Conference on Genetic Variation of Viruses sponsored by the New York Academy of Sciences, New York, November 28-30, 1979
Invited Seminar Convener, Viral Vaccines of the Future, American Society for Microbiology meeting, May 11-16, 1980, Miami Beach, Florida

Harry B. Greenberg

Invited speaker, XI meeting of Perspectives in Virology, February, 1980, New York

Anthony R. Kalica

Invited speaker, Thirteenth Joint Conference on Viral Diseases, United States-Japan Cooperative Medical Science Program, Atlanta, Georgia, October, 1979

Stephen M. Feinstone

Invited participant, Basel Liver Week Symposium on Viral Hepatitis, Basel, Switzerland, October, 1979
Invited participant, 2nd Australian Symposium on Viral Hepatitis, Melbourne, Australia, March, 1980
Invited particpant, 1st International Symposium on Non-A, Non-B Hepatitis, Vienna, Austria, June, 1980

Vincent J. McAuliffe

Invited speaker, American Society for Microbiology meeting, May, 1980, Miami Beach, Florida

Richard G. Wyatt

Consultant to Navajo Community College, Tsaile, Navajo Nation (Arizona) on Minority Biomedical Support Project: "Gastroenteritis in Newborn Lambs and Young Children on the Navajo Reservation", June 19-22, 1979
Invited speaker, Symposium on Viral Enteritis in Humans and Animals, Grignon, France, September 4-7, 1979
Invited speaker, Thirteenth Joint Conference on Viral Diseases, U.S.-Japan Cooperative Medical Science Program, Atlanta, Ga., October 1-3, 1979
Consultant on U.S.-Egypt PL480 Project "Etiology of Viral and Nonviral Diarrheas (03-440-C), 1980
Invited presentation on Viral Diarrhea to students participating in the Minority Biomedical Support Program, NIH, February 28, 1979 and February 12, 1980

Lewis J. Markoff

Invited speaker, International Workshop on Structure and Variation of Influenza Virus, Tredbo, Australia, December 10-12, 1979

Ching-Juh Lai

Invited speaker, Conference on Genetic Variation of Viruses, New York Academy of Sciences, November 28-30, 1979
Invited speaker, International Workshop on Structure and Variation of Influenza Virus, Tredbo, Australia, December 10-12, 1979

Ching-Juh Lai (continued)

Invited speaker, Biology Department, Molecular Biology Institute, U.C.L.A., Los Angeles, California, May 2, 1980

Invited speaker, Seminar on Viral Vaccines of the Future, American Society for Microbiology, Miami Beach, Florida, May 11-16, 1980

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00021-11 LID

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

THE ETIOLOGY OF ACUTE INFECTIOUS NONBACTERIAL GASTROENTERITIS

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. Albert Z. Kapikian	LID, NIAID	Head, Epidemiology Section
	Dr. Harry B. Greenberg	LID, NIAID	Medical Officer
	Dr. Anthony R. Kalica	LID, NIAID	Research Microbiologist
	Dr. Richard G. Wyatt	LID, NIAID	Medical Officer
	Dr. Jorge Flores	LID, NIAID	Expert Consultant
Other:	Dr. Robert M. Chanock,	LID, NIAID	Chief, LID, NIAID
	Dr. Nalini Singh	LID, NIAID	Visiting Associate
	Mr. W. Lee Cline	LID, NIAID	Bio. Lab. Tech. (Micro)

COOPERATING UNITS (if any)

see separate page

LAB/BRANCH
LABORATORY OF INFECTIOUS DISEASES

SECTION
EPIDEMIOLOGY SECTION

INSTITUTE AND LOCATION
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES, BETHESDA, MARYLAND

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
11.9	4.58	7.33

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Objectives: (1) To search for viruses which play an etiological role in the syndrome of acute infectious non-bacterial gastroenteritis of infants, children, and adults; (2) to cultivate (in vitro) the viral agents of acute infectious non-bacterial gastroenteritis; (3) to study the biophysical, biochemical, genetic, immunological and epidemiological characteristics of such agents; (4) to reproduce the syndrome experimentally for the purpose of a) studying the pathophysiological and immunological responses of the host, and b) assaying the infectivity of viruses such as human rotaviruses and the Norwalk and Norwalk-like agents; (5) to develop effective immunoprophylaxis for the viruses of acute, infectious, non-bacterial gastroenteritis; (6) to develop efficient and sensitive assays for a) detection of the viral and bacterial agents associated with gastroenteritis and b) antibodies to these pathogens.

Cooperating Units

Children's Hospital National Medical Center, Washington, D.C.

Johns Hopkins University, Baltimore, Maryland

Division of Geographic Medicine of the Johns Hopkins University School of Medicine and the Baltimore City Hospitals, Baltimore, Maryland

Center for Disease Control, Atlanta, Georgia

Meloy Laboratories, Rockville, Maryland

Plum Island Animal Disease Center, USDA, New York

University of Minnesota School of Medicine

University of Massachusetts

Bureau of Biologics, FDA; NINCDS; Veterinary Resources Branch, DRS

Cholera Research Laboratory, Dacca, Bangladesh

INISA San Jose Costa Rica

INCAP, Guatemala

Depaertment of Veterinary Science, Ohio Agricultural Research and Development Center, Wooster, Ohio

Laboratory of Streptococcal Diseases, NIAID

Center for Vaccine Development, University of Maryland, Baltimore, Maryland

University of Rochester, New York

State Health Department, Berkeley, California

ICRR Syndney, Australia

Institute of Biomedical Investigations, Mexico City, Mexico

Major Findings

Background - Important advances have been made in elucidating the etiological agents of acute infectious nonbacterial gastroenteritis, a syndrome that affects a broad segment of the population. It appears that viral gastroenteritis consists of at least two entities with distinct epidemiological characteristics. One, designated epidemic viral gastroenteritis, tends to occur in family or community-wide outbreaks affecting adults, school-aged children, family contacts and probably young children as well. The illness is usually self-limited and characteristically lasts 24-48 hours. In 1972, we demonstrated by immune electron microscopy (IEM) that a 27nm particle -- the Norwalk agent -- was associated with an outbreak of this form of gastroenteritis that occurred in Norwalk, Ohio. In addition, in further studies by our laboratory, particles that resemble the Norwalk agent morphologically have also been associated by IEM with 2 family outbreaks of gastroenteritis, one in Hawaii and the other in Montgomery County (MC), Md. The Norwalk and Hawaii agents appear to be distinct, whereas the Norwalk and MC agents appear to be related. An agent designated Ditchling or "W" has been associated with this form of gastroenteritis in studies in England. In addition, other "small round viruses" some with calcivirus-like morphology have been associated with outbreaks of gastroenteritis in various parts of the world.

The other form of gastroenteritis, designated sporadic infantile gastroenteritis, represents a severe form of diarrhea affecting infants and young children, that often necessitates hospitalization and parenteral fluid therapy. It is evident now from studies in many parts of the world that the 70nm human rotavirus is the major known etiological agent of sporadic infantile gastroenteritis. Before the discovery of this agent by Australian investigators in 1973, the etiology of the majority of cases of infantile gastroenteritis was unknown.

Our gastroenteritis studies are focused on the rotaviruses as well as on the Norwalk group of viruses. The Norwalk group of agents has not been cultivated successfully in any *in vitro* system. While human rotaviruses are capable of initiating infection of cells in culture the infective process is abortive and infectious virus is not produced. However, in spite of the failure to grow the Norwalk agent, our studies have elucidated the importance of the Norwalk virus as a cause of epidemic viral gastroenteritis in various epidemiologic settings. In addition, a major breakthrough was described in last years annual report - the successful serial propagation of human rotavirus type 2 *in vitro*. Highlights of research activities carried out with LID staff alone or in collaboration with others are outlined below.

Continued Studies of Human Rotavirus Type 2 In Vitro.

In last year's annual report we described the successful cultivation of the "Wa" strain of human rotavirus type 2 *in vitro*. Our ability to propagate type 2 human rotavirus in culture made it possible for us to characterize this fastidious agent in greater detail than heretofore possible and to study its antigenic properties as they relate to homotypic as well as heterotypic immunity. An efficient plaque assay was developed for quantitating virus and isolating genetically homogeneous populations of virus. In addition, optimal conditions were defined for growing virus in a cell substrate suitable for propagation of virus to be administered to man. Plaque purified virus is currently being grown in African green monkey kidney (AGMK) cell culture and will be evaluated for its virulence in susceptible adult volunteers with low or undetectable intestinal secretary IgA rotavirus antibody. If the culture adapted type 2 human rotavirus proves to be attenuated in this situation it will be tested in successively younger individuals for its suitability as an attenuated vaccine strain. Plaque-purified "Wa" strain grown in AGMK culture administered to two germfree calves by the intraduodenal route did not produce disease suggesting that the tissue culture adapted human rotavirus may be attenuated.

A plaque reduction assay for measurement of serum neutralizing antibody was developed using CV-1 or MA104 cells and an agarose overlay containing trypsin. Using this plaque reduction antibody system, we confirmed the results of previous immunofluorescence studies that showed the "Wa" virus to be antigenically distinct from bovine, porcine, and simian rotaviruses. Additional hyperimmune guinea pig sera and post-infection sera from gnotobiotic calves were evaluated for their ability to distinguish the "Wa" strain from the tissue culture adapted animal rotaviruses. The most potent and specific of these sera were found to be very useful in characterizing newly-isolated reassortant viruses containing a mixture of human and animal rotavirus genes permitting us to identify those reassortants which possessed the "human" outer capsid protein that reacted with neutralizing antibody.

Ten different types of primary cell culture and 14 cell lines were tested for their ability to support growth of the "Wa" strain; efficient plaque formation occurred only in primary African green monkey kidney, CV-1 and MA104 cells. Cynomologous monkey kidney and calf kidney cells as well as LLC-MK_2, Buffalo green monkey kidney, HRT-18, and BSC-1 cell lines supported the growth of the "Wa" less well but were not useful for plaque assay.

Finally, a reference suspension of tissue culture grown type 2 human rotavirus was prepared and distributed to at least 27 different investigators in the USA and other countries.

Etiology of Non-Bacterial Gastroenteritis Among Infants and Young Children Admitted to Children's Hospital National Medical Center, Washington, D.C. - These studies are carried out collaboratively with Children's Hospital National Medical Center, Washington, D.C. Currently stool or rectal swab speciemns are examined by electron microscopy (EM) and ELISA at Children's Hospital for gastroenteric viral agents (Dr. Brandt), whereas rotavirus serologic studies (CF) are carried out at the LID (Mr. Cline). The clinical data and specimens are furnished by Dr. Kim and her staff of Children's Hospital. The bacterial studies are performed by the staff of Children's Hospital (Dr. Rodriguez). In addition, serotyping of rotaviruses is performed at LID. The scope of the study was outlined previously and essentially involved study of pediatric patients with a diarrheal illness admitted to Children's Hospital. Limited outpatient studies were also performed. Data from this study were summarized in previous annual reports. The winter season peak of hospitalization for diarrhea associated with rotavirus was again observed at Children's Hospital in 1980 although the peak occurred slightly later than previously. Serotyping of rotaviruses is currently underway in LID using the newly developed IAHA test as well as the previously described ELISA. Serologic studies of paired sera from hospitalized patients are also planned.

Studies on the Etiology of Non-Bacterial Gastroenteritis Among Family Members in a Suburban Virginia Community. - These studies are under the supervision of Dr. William J. Rodriguez of Children's Hospital National Medical Center and are aimed at elucidating the incidence and etiologic agents of gastroenteritis in family members in suburban Virginia. Examination of stools or rectal swabs for rotavirus and bacterial pathogens is carried out at Children's Hospital, whereas serologic studies for antibody response to rotavirus and Norwalk virus are prformed in LID. Detailed analysis of this study will be included in next years annual report.

Development of an ELISA for Detection of Adenovirus - An ELISA was developed for detection of adenovirus in stool or rectal swab specimens. This if of more that academic interest since noncultivatable or fastidious adenoviruses have been associated with a small, but consistent proportion of gastroenteric illness of infants and young children. In the ELISA, a pre-immunization or hyperimmune guinea pig serum to adenovirus type 2 is employed to precoat the wells of a microtiter plate. The hyperimmune serum functions as a capture antibody, while the pre-immunization serum serves as a specificity control since it does not contain adenovirus antiboy. The detection antibody is a commercially available goat antiserum to adenovirus type 2 (Microbiological Associates, Inc.). The conjugate is a commercially available rabbit anti-goat globulin antibody linked to alkaline phosphatase (Northeast Biomedical Laboratories). The substrate is p-nitrophenyl phosphate (Sigma). For a specimen to be considered adenovirus positive, the well pre-coated with hyperimmune serum must have an optical density at least 50% greater than a well precoated with the pre-immunization serum. The test appears to be specific and sensitive for detection of adenovirus. Correlation of the ELISA with EM examination of stool or rectal swab specimens for adenovirus is in progress. Thus far, 369 specimens from Children's Hospital have been examined by both methods. The adenovirus ELISA is capable of detecting viruses belonging to each of the 31 serotypes tested. However, its greatest usefulness lies in its ability to detect non-cultivatable adenoviruses which appear to be the major enteric pathogens of this virus family.

Seroepidemiology of Rotavirus Infection in Rural Bangladesh

This study was carried out in collaboration with Drs. Sack, Gilman, and Aziz with the LID performing the serological tests (CF). A seroepidemiological study of rotavirus infection was performed in children in a village in rural Bangladesh. The serum specimens studied were collected from 85 children, 0-4 years of age, who lived in one village and who were bled at 3 month intervals over the period May, 1976-September, 1977. Fifty-seven of the 85 children developed a rise in serum rotavirus antibody; 9 of the study subjects developed two separate seroresponses. Winter infections were most frequent, although a large cluster was observed during a summer monsoon season.

Epidemiological Studies of Diarrheal Illness in Hospitalized Infants in Egypt - As a part of a collaboration with Egyptian and University of Texas scientists under a PL 480 agreement, a pilot study was carried out at Cairo University Children's Hospital between October and December 1979. Sixty two cases of diarrheal illness were studied for rotavirus and adenovirus infection by ELISA in LID. Nineteen patients 19 (31%) were positive for rotavirus; in contrast one (3%) of 33 controls shed rotavirus shed rotavirus. Adenovirus was detected in 2 of 62 patients, but not in any of the controls. Dr. Tary Naguib, a Guest Worker from Egypt, was instructed in the enzyme immunoassay procedures needed for the projected longitudinal studies to be performed in Cairo.

Study of Rotavirus Infection in Adult Volunteers

This study was summarized in detail in last year's annual report. However, one additional finding was made when the data was subjected to additional statistical analysis. The absence of detectable serum immunofluorescence antibody for rotavirus correlated with susceptibility to the development of illness following rotavirus challenge; however, as noted previously the prime correlate for resistance to illness following challenge appeared to be local intestinal IgA rotavirus antibody.

Development of an Immune Adherence Hemagglutination Assay (IAHA) Test for Serotyping Rotavirus

An IAHA test was developed for the purpose of serotyping human rotavirus types 1 and 2 as well as certain animal rotaviruses. In this test, convalescent phase serum from a gnotobiotic calfe infected with human rotavirus type 1 or 2 was employed as the source of antibody. This test system has enabled us to serotype most human rotavirus strains as well as reassortants produced by mixed infection of human and animal rotavirus.

Preparation of Rotavirus Reference Reagents.

LID participated in the preparation and certification of seven reagents to be distributed through the Research Resources Branch/NIAID, WHO, and PAHO, for the purpose of diagnosing rotavirus chiefly by enzyme immunoassay.

These reagents include pre-and hyperimmune guinea pig serum, pre- and hyperimmune goat serum, and pre- and post-infection calf serum against human rotavirus; in addition a human rotavirus (type 2) positive antigen was prepared from the feces of an infected gnotobiotic calf.

Further Studies of Bovine Rotavirus as a Potential Human Vaccine

The ability of bovine rotavirus to protect newborn calves against subsequent challenge with human rotavirus was described initially in the FY 1978 annual report and in greater detail in last year's annual report. In addition, the preparation of a suspension of the UK strain of calf rotavirus suitable for study in man was described in last year's annual report. It was also noted that the UK strain of bovine rotavirus induced illness in newborn calves which was similar to that produced by U.S. bovine NCDV strain. However, the latter did not induce illness in gnotobiotic piglets, whereas the former induced serious disease in these animals. The tissue culture grown UK strain was tested further in additional gnotobiotic piglets and its virulence was compared to that of the oroginal vius strain which had not been passaged in cell culture. Both the plaque-purified virus and the original UK virus (tested as a suspension of calf feces) produced lethal disease in piglets. Because the U.S. strain of calf diarrhea virus (NCDV) does not produce disease in piglets, relaxation of restrictions on the UK rotavirus is unlikely thus limiting efforts to evaluate the UK virus as a potential live vaccine for man.

Development of Temperature-Sensitive ts Mutants of Animal Rotaviruses

As part of our effort to investigate and modify the virulence of rotaviruses, 10 *ts* mutants of the UK strain of calf rotavirus were isolated after mutagenesis with 5 azacytidine and/or 5 fluorouracil. Each of the mutants had a shutoff temperature for plaque formation of 38°C or 39°C and each was relatively stable genetically. Analysis of the ten mutants (M1, 2, 4, 5, 6, 7, 8 10, 11, 13) showed that they belonged to four separate, non-overlapping recombination groups. Polyacrylamide gel analysis of the RNA of these mutants indicated that each exhibited the same pattern as the *ts*$^+$ wild type UK parent. In addition, we recently isolated 6 *ts* mutants of the SA-11 simian rotavirus which fell into 3 non-overlapping recombination groups.

Use of ts Mutants to Rescue Non-Cultivatable Human Rotavirus by Gene Reassortment

Except for the "Wa" strain of type 2 human rotavirus, human rotaviruses have not been successfully propagated serially *in vitro* to high titer. In addition, most animal and avian rotaviruses are extremely fastitious and cannot be cultivated *in vitro* by current cell culture techniques. For these reasons we developed a strategy for converting fastidious rotaviruses into viruses capable of efficient growth in tissue culture. This was achieved by genetic reassortment during mixed infection with a cultivatable rotavirus. In this manner, the genes of the fastidious rotavirus which restricted growth *in vitro* were replaced by the corresponding genes from a tissue culture adapted rotavirus. In the first experiments we used ts mutants of the tissue culture adapted UK or SA-11 virus to rescue type 1 or 2 non-cultivatable human rotavirus. Briefly, a non-cultivatable strain of human rotavirus which had been amplified by passage in gnotobiotic calves or which was present in high titer in human stool (Wa) was centrifuged at low speed onto AGMK cells. The cells were then superinfected with a ts mutant of a cultivatable bovine or simian rotavirus (UK or SA11) and the infection was allowed to proceed for 36 hours at 34°C. The infected cell culture harvest was then plated out at restrictive temperature with or without UK or SA11 antiserum. Using this technique, we recovered reassortant viruses that grew to high titer and were neutralized specifically by hyperimmune guinea pig type 1 or type 2 human rotavirus antiserum. Preliminary RNA analysis of these clones disclosed that they were indeed reassortant viruses. It was of interest that the neutralization and IAHA serotype specificities appeared to reassort independently suggesting that the capsid protein reacting with neutralizing antibodies differs from that measured in the serotyping reaction. Gene rescue should prove useful in propagating other non-cultivatable human rotaviruses as well as fastidious animal rotaviruses such as epidemic disease of infant mice (EDIM). Appropriate analysis of such reassortants should allow us to construct a map of the rotavirus genome and to identify the location and function of each of its gene products.

Rescue of the non-cultivatable type 1 human rotavirus by co-infection with a cultivatable bovine rotavirus yielded reassortants that grew to high titer in tissue culture, produced plaques with high efficiency and were specifically neutralized by hyperimmune type 1 human rotavirus antiserum. It should be emphasized that this achievement provides us with a mechanism for generating cultivatable type 1 virus that can be used for immunoprophylaxis in man. This is of some importance since the procedure previously used for in vitro cultivation of the type 2 human rotavirus was not successful for type 1 virus. A cultivatable mutant of type 2 virus emerged during serial passage of the virus in gnotobiotic piglets. In contrast type 1 virus could not be passaged serially in piglets and for this reason another strategy for growing the virus in tissue culture was needed. Analysis of type 1 human rotavirus reassortants that grow well in cell culture should allow us to identify the genes that restrict in vitro propagation of naturally occurring type 1 viruses

Studies with Rotaviral RNA

(A) Differences in Electrophoretic Mobility of Rotavirus dsRNA as an Aid in Identifying Serotype and Characterizing Strains from Different Species- Our previously published RNA pattern of DS-1 strain of type 1 human rotavirus (from Children's Hospital National Medical Center) was found to be incorrect as the result of a laboratory error. The correct pattern for DS-1 was similar to that found by Dr. Espejo for one of two groups of rotaviruses recovered from patients in Mexico City. In collaboration with Dr. Espejo a possible correlation of RNA pattern with

ELISA serotype among human rotavirus isolates was sought. Three serotype 1 strains including DS-1, Bangladesh #268 and HRV 2017 (from Mexico City) had an RNA pattern with slow migrating 10th and 11th segments and this distinguished them from nine serotype 2 viruses one of which was from Mexico City (HRV 4185). More strains will be examined to confirm this correlation. If the correlation is confirmed it might provide an alternative to serotyping which is currently somewhat limited by the lack of serotyping reagents.

The RNA's from two newly isolated rhesus monkey rotaviral strains were found to be distinct from simian rotavirus strain SA11. The former strains are potential vaccine strains and characterization of their biological, biochemical and biophysical properties is planned.

One approach to the construction of a genetic map of the human rotaviruses involves an analysis of the appropriate human-animal rotavirus reassortants. For this approach to suceed it is necessary to be able to distinguish each of the corresponding genes of both parents by gel electrophoresis. Recently a discontinuous system with greater resolving power was developed. Using this method six of the seven smallest genes of the UK bovine rotavirus and the "Wa" type 2 human rotavirus could be distinguished. With further modification it is likely that each of the eleven genes of "Wa" can be distinguished from the corresponding gene of the UK bovine virus.

(B) In vitro transcription - Virion RNA polymerase activity has been studied extensively in reoviruses and more recently in animal rotaviruses. We have studied this enzyme in preparations of "Wa" and UK cores. Treatment of the virus with EDTA activates the enzyme which can be assayed by measuring the incorporation of label from ^{32}P-ATP or ^{3}H UTP into RNA. Optimal conditions have been established for production of complete or nearly complete copies of each of the viral genes. The single stranded RNA synthesized during this reaction functions as "messenger RNA" in an "in vitro" translation system; it also hybridizes with single stranded RNA obtained by denaturing the corresponding viral double stranded (ds)RNA.

Viral transcripts ("mRNA") may prove helpful in identifying the protein coded by each of the rotavirus genes. Thus far, we have translated 5 proteins from "Wa" and four from UK utilizing a rabbit reticulocyte system. The background protein synthesis occurring in this system (in the absence of externally added mRNA) has obscured detection of other viral proteins. Specific immunoprecipitation of viral proteins produced in the cell free system resolve this problem.

We plan to use ^{32}P labelled ssRNA to genotype reassortants obtained from simultaneous infection with "Wa" and UK viruses. ^{32}P ssRNA from UK or "Wa" will be hybridized with RNA obtained from each one of the reassortants. Electrophoresis and autoradiography of the resulting hybrids should yield information about the parental origin of each gene in each reassortant.

Rotaviral Polypeptides

(A) Labelling with S-35 methionine - We have continued to study rotaviral polypeptides in order to provide: i) better biochemical characterization of rotaviruses; ii) a basis for comparison with rotaviral gene products which are produced by translation of denatured ds RNA or RNA transcripts; iii) a basis for comparison of possible mutagen induced or naturally occurring mutations which affect the polypeptides and; iv) an understanding of which rotaviral proteins are important in immunity.

The proteins of the human rotavirus "Wa" strain and UK calf virus were studied by incorporation of ^{35}S methonine during infection in MA104 or CV-1 cells. Background cellular protein synthesis decreases resolution of viral proteins. This difficulty was overcome in part by use of immunoprecipitation.

An alternate method which proved more successful in increasing resolution of viral proteins produced in cell culture involved the use of a high concentration of NaCl in culture medium during the labelling period. This modification of technique reduced background cellular protein synthesis. When the high salt method was used we were able to identify 10 human rotaviral polypeptides in cytoplasmic extracts; these polypeptides ranged from 16,800 to 128,000 daltons. Seven of these were structural proteins; two of the 7 sere located in the outer capsid of the virus.

There were eleven UK calf polypeptides in cytoplasmic extracts of 18 hour infected MA104 cells and these proteins ranged from 12,000 to 132,000 daltons. Identification of the UK structural polypeptides and their location in the virion is currently underway.

Because it grows to high titer and possesses a stable hemagglutinin, SA11 virus was used to compare the production of polypeptides during infection following a multiplicity of infection (MOI) of 100 (10-fold above that used for "Wa" or UK) under conditions of increased salt concentration. Thirteen SA11 polypeptides, ranging from approximately 10,500 to 130,000 daltons were labelled. Two of the thirteen polypeptides were not seen when increased NaCl was used to reduce background. Efforts are planned to increase the titer of human rotavirus comparable to that used with SA11 to determine if additional polypeptides can be detected with a higher MOI. Seven of the 13 SA11 polypeptides were structural and two of the seven were on the outer capsid of complete hemagglutinating SA11 virions.

The polypeptides of SA11 were also labelled using ^{14}C amino acids instead of ^{35}S methionine. Labelling with ^{14}C amino acids yielded results identical to those obtained with ^{35}S methionine.

(B) Glycoproteins - The relevant role of structural glycoproteins in viral pathogenesis, antigenicity and host immunity has been recognized for many viruses. We have employed several radioactively labelled carbohydrates to determine which of the different human and animal rotavirus polypeptides are glycosylated. Different cell types (MA 104, AGMK, CV1) are infected with virus. After a 1 hour period of carbohydrate deprivation and a 1 hour period of labelling with the chosen carbohydrate the infected cells are washed, lysed and the products subjected to SDS acrylamide electrophoresis. The glycosylated polypeptides are then resolved by fluorography. Labelling with ^{14}C-glucosamine seems to be the most effective way of identifying rotavirus glycoproteins. Incorporation of this carbohydrate occurred early in infection (3 hr) and followed closely the synthesis of the viral proteins that were glycosylated. A longer incubation (18-24 hr) allowed more distinct identification of specific viral glycoproteins since cell protein synthesis was strongly inhibited late in the infection.

Current findings indicate the presence of at least three glycosylated viral proteins in cells infected with the human "Wa" strain. One protein with a weight of 40,000 Daltons seems to be an inner capsid component and the most abundant viral polypeptide. The location of the other two glycoproteins in the virus has not been determined due to difficulties in obtaining enough ^{14}C-glycosylated complete virus.

Surprisingly, infection of cells with an animal rotavirus such as SA11, NCDV or UK yielded up to seven clearly distinguishable viral glycoproteins. Three proteins seemed to correspond to large inner capsid proteins.

The specificity of glycosylation and its significance are under current investigation utilizing two different approaches: a) immunoprecipitation of ^{14}C-glycosylated proteins with specific antiserum followed by staphyloccal protein A and electrophoresis and autoradiography; b) identification of glycoproteins in non-labelled purified virus by blotting ts proteins (separated by electrophoresis) on a nitrocellulose filter and labelling them with ^{125}I concanavalin A, which should specifically bind to glycosydic residues.

Development of Hybridomas For Human Rotavirus

Purified "Wa" virus was used to develop mouse hybridomas against the human rotavirus. Thus far, three antibody producing clones have been identified. Antibody production was assessed by RIA and immunofluorescence.

Role of Norwalk Virus in Outbreaks of Non-Bacterial Gastroenteritis

The development of a radioimmunoassay (RIA) for detection of Norwalk antigen and a radioimmunoassay blocking (RIABL) test for detection of antibody to the Norwalk agent were described in previous annual reports. The availability of the RIA and RIABL has permitted continued epidemiological studies of Norwalk infection.

Our survey of outbreaks of gastroenteritis was extended during the past year. To date approximately 75 separate outbreaks have been studied serologically for Norwalk infection and approximately one third appeared to be associated with the Norwalk virus. The latter group includes several well-documented waterborne outbreaks and 2 oyster associated outbreaks (one in Australia and one in Florida).

In collaborative studies with Dr. W. Rodriguez, Children's Hospital National Medical Center, Washington, D.C., Dr. Mark Gurwith, UCLA Medical School, Dr. R. Ryder, Gorgas Hospital, Panama and Dr. R. Black, formerly of CRL, Bangladesh we have also studied the incidence of Norwalk infection in various populations using RIABL. Preliminary data show that infection with Norwalk virus is extremely common in Bangladesh with an approximate 50% seroconversion rate in children between one and two years of age. Association of Norwalk infection with illness in this population is being analyzed. In Panama, Washington, D.C. and Winnepeg, Canada, Norwalk infection appears to be less common. In Canadian Eskimos and Indians the infection rate with Norwalk virus is considerably higher than in Winnepeg residents but still considerably less than encountered in Bangladesh. Study of 30 seriously ill children in Bangladesh showed that Norwalk virus was an infrequent cause of severe gastroenteritis in this group. In summary, preliminary data indicates that Norwalk virus may, at least in some populations, be a common cause of mild endemic infantile gastroenteritis.

Purification of the Norwalk Antigen

We have recently purified Norwalk virion and a soluble viral protein found in the stools of infected individuals. The virion was purified by a combination of fluorocarbon extraction, isopycnic banding in cesium chloride and renographin and rate zonal sedimentation in sucrose. The specificity of the proteins released from dissociated, iodinated, purified virions was determined by immunoprecipitation using paired sera from volunteers infected with Norwalk virus. The soluble virion protein found in stool was purified by gel chromatography. The major structural protein of the Norwalk virus has a molecular weight of approximately 59,000 daltons. This finding suggests that the Norwalk virus is most likely a calicivirus, rather than a parvovirus as thought previously. The soluble viral protein found in stool has a molecular weight of approximately 30,000 daltons and its relationship to virion protein is unclear.

Other Agents

In last year's annual report we described studies with another 27 nm particle, the Marin County agent (kindly supplied by Dr. L. Oshiro). We noted that this agent, which was clearly associated with an outbreak of gastroenteritis in a nursing home in California by Dr. Oshiro, was not serologically related to the Hawaii or Norwalk agents. We also noted that we had given a stool filtrate preparation of the agent to two chimpanzees and that neither became ill or seroconverted by IEM.

The Marin County virus suspension, which was free of detectable adventitious agents, was administered to two volunteers. One developed mild gastroenteritis. Neither volunteer shed viral particles detectable by IEM. The IEM serum antibody response of the ill volunteer is currently being evaluated.

Crohn's Disease

Approximately 50 patients with Crohn's disease were studied prospectively to determine if intercurrent gastrointestinal viral infection was associated with exacerbation of disease. None of the patients shed rotavirus, adenovirus or Norwalk virus during the study. There were no seroresponses to Norwalk virus and only rarely to rotavirus. These findings indicate that intercurrent viral infections are not a common contributor to disease activity in inflammatory bowel disease.

In another study with Drs. Gump, Caul, Eade, MacPherson, Mitchell, Parent, Richmond, and Becker in which the Norwalk and rotavirus serologic studies were performed at LID, the prevalence of antibody to Norwalk virus or rotavirus was not significantly greater in Crohn's disease patients than in matched controls. These investigators found an increase in antibody prevalence to Pseudomonas-like bacterium and Bacteriodes vulgatus but not to Chlamydia trachomatis in Crohn's disease patients. In addition, they found increased lymphocytotoxic antibody titers in patients, but these titers did not correlate with the titers of any of the microbial agents tested.

Study of the Epidemiology of Epstein-Barr Virus Infection - These studies were carried out by Drs. Chang and Rosenand were essentially completed last year but are being described in this year's annual report. The LID furnished serum specimens previously obtained from residents of Junior Village--a welfare home for homeless but otherwise normal children.

Tests for Epstein-Barr virus (EBV) capsid antibody were carried out on 115 infants and young children on admission and discharge. Forty-three children positive on admission remained positive at discharge, 44 of 72 negative on admission remained negative at discharge, and 28 of 72 negative on admission converted to positive at discharge.

Age, sex and season did not appear to influence the rate of EBV-seroconversion among the nursery children. The only identifiable factor that significantly increased the rate of EBV-seroconversion was the duration of nursery residency. The rates were 1/9 (11%) 1/19 (3%) and 11/16 (69%) among children residing for 1.5 to 2.4, 2.5 to 4.4 and 4.5 to 7.4 months respectively. Children residing in the nursery for 1.5 to 4.4 months and children residing at home had similar EBV-seroconversion rates. For children residing in the nursery for 4.5 to 7.4 months, however, the EBV-seroconversion rate was much higher than the rate for children living at home.

Relevance of Project to Missions of NIAID

Acute infectious nonbacterial gastroenteritis is a common infectious disease which affects a broad segment of the population and was the second most common disease observed in a 10 year family study in the United States. In addition, diarrheal diseases are a leading cause of mortablity as well as morbidity in developing countries. Ultimate goals of prevention and therapy have been furthered in our current studies by (i) the detection and preliminary characterization of etiology agents of this disease, (ii) the development of sensitive, efficient and rapid assay systems by which the epidemiologic importance of the diseasecan be determined, (iii) the *in vitro* cultivation of type 2 human rotavirus, (iv) development of a type 1 human rotavirus reassortant that grows well in cell culture, (v) the ability to induce illness in human volunteers and experimental animals administered the human rotavirus, (vi) the elucidation of the importance of intestinal fluid IgA rotavirus antibody in preventing rotavirus illness under experimental conditions, (vii) development of biophysical and serologic methods to differentiate the human rotavirus from other related agents and (viii) the elucidation of the epidemiology of the rotavirus and Norwalk viruses.

Proposed Course of Project

In future studies of the Epidemiology Section, major emphasis will be given to the efficient propagation of the viral gastroenteritis agent *in vitro*, to the delineation of their overall importance over a sustained period in the etiology of gastroenteritis in various populations, to the immune mechanisms involved in host-defense, to the development of effective methods to prevent illness due to these agents, and to the continued search for other etiologic agents of acute infectious gastroenteritis.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00024-20 LID

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

LABORATORY STUDIES OF MYXOVIRUSES

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Brian R. Murphy, M.D.	Medical Officer	LID, NIAID
	Mark D. Tolpin, M.D.	Research Associate	LID, NIAID
	Robert M. Chanock, M.D.	Chief	LID, NIAID

COOPERATING UNITS (if any)

Flow Laboratories (contract # N01 AI 32521)
NINCDS; Bureau of Biologics, FDA, National Cancer Institute, Immunology Branch

LAB/BRANCH LABORATORY OF INFECTIOUS DISEASES

SECTION RESPIRATORY VIRUSES SECTION

INSTITUTE AND LOCATION

NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES, BETHESDA, MARYLAND

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

ts-P3 and ts-P1 mutations in ts-1A2 recombinant viruses specified a satisfactory level of attenuation of influenza A viruses in man. Suppressor and other mutations resulted in loss of ts phenotype of a ts-1A2 recombinant during viral replication in a completely susceptible child. The mutation that suppressed the ts-P3 gene was located on the P2 gene. A new "ts-1[E] like "donor" virus containing ts-P3 and ts-NP genes was produced and found to be attenuated and genetically stable in hamsters. Characterization of the ts phenotype of A/AA/6/60 ca recombinants suggested that attenuation of the ca recombinants in man is not dependent solely on the ts property. ELISA's were developed to quantitate IgG, IgA, and IgM influenza A or B virus hemagglutinin antibodies in serum and nasal wash specimens. Squirrel monkey model of influenza A virus infection was extended to permit repeated sampling of the lower respiratory tract for virus without injury to the animal.

Project Description

Characterization of ts-1A2 Recombinant Viruses

The A/Udorn/72 ts-1A2 virus with ts mutation(s) on the P3 and P1 genes has been evaluated as a donor of these ts genes to new variants of influenza A virus. The work this year involved the genotyping of fifteen A/Alaska/77-ts-1A2 (H3N2) and eleven A/Hong Kong/123/77-ts-1A2 recombinants. Genotype of recombinant viruses was determined by measuring the migration rate of virion RNA from parental and recombinant viruses on polyacrylamide gel or by antigenic analysis using conventional antiserum or monoclonal antibody. The latter analysis was performed in collaboration with Dr. Kathleen Van Wyke of St. Jude Hospital in Memphis, Tennessee. The genotyping of the A/Alaska/77-ts-1A2 recombinants confirmed our assignment of the locus of the ts genes that had been established previously by complementation-recombination analysis. However, the genotyping of the A/Hong Kong/77-ts-1A2 recombinants yielded several surprises. Two viruses which were thought to have received the P1 ts gene from the ts-1A2 virus based on complementation analysis had a wild type gene at that locus. One of these viruses (ts-127) had genes from the wild type parent at all eight loci indicating that it was a spontaneous ts mutant. This virus failed to complement the prototype ts P1 virus, but complemented all of our other ts probes. These results suggested that a spontaneous ts mutation occurred on the P1 gene. The situation was further complicated by the observation that this putative ts P1 mutant underwent complementation with segregants of a ts-1A2 virus that contained a ts-P1 gene. Another ts virus(ts-104) that behaved like ts 127 was a recombinant virus with ts-1A2 genes at the M and NS loci and a P1 gene derived from wild type virus. Considered together these results suggest that ts-127 and ts-104 viruses underwent intercistronic complementation with ts 1A2 segregants containing a ts P1 gene of ts-1A2 origin. Currently the locus of the ts lesion of the ts-127 and ts-104 viruses is being determined by genotyping recombinants derived from these viruses.

The major finding derived from the genotyping of the ts-1A2 recombinants was the demonstration that the two ts-1A2 ts genes were the determinants of growth restriction of ts-1A2 recombinants in animals and of attenuation for man. In one instance, only the ts genes of the ts-1A2 donor were transferred into a recombinant bearing H1N1 surface antigens. This recombinant was satisfactorily attenuated for doubly seronegative (susceptible) individuals indicating that the two ts-1A2 ts genes (P1 and P3) can act as the sole determinants of attenuation. The two ts genes were capable of producing the desired level of attenuation of a wild type virus without the cooperation of other genes from the ts-1A2 donor virus. The observation that three influenza A viruses belonging to two subtypes could be satisfactorily attenuated by the transfer of the two ts-1A2 ts lesions supported the hypothesis underlying our approach to the production of live influenza A virus vaccines, namely wild type virus can be attenuated to a specific desired level by the introduction of genes bearing defined ts mutations.

Since the A/Alaska/77-*ts*-1A2 virus lost its *ts* phenotype after replication in a doubly-seronegative child, it was important to determine if a modifying mutation had occurred during the production of this A/Alaska/77-*ts*-1A2 recombinant that might have caused a loss in genetic stability. This was important to explore since the A/Victoria/75-*ts*-1A2 (H3N2) and A/Hong Kong/77-*ts*-1A2 (H1N1) recombinants had not lost their *ts* phenotype after replication in a limited number of doubly seronegative individuals. An *in vitro* stress test was developed in which the donor or recombinant *ts*-1A2 virus was grown at semi-restrictive temperature and the progeny viruses analyzed for their level of temperature-sensitivity. Progeny of the *ts*-1A2 donor and recombinant viruses that were grown at 37°C exhibited a 39°C shutoff temperature for plaque formation. Further passages at 38°C or 39°C led to the emergence of *ts*$^+$ virus with a 40°C shutoff temperature. Importantly, donor and recombinant *ts*-1A2 viruses behaved similarly in the *in vitro* stress test. These results suggested that emergence of a *ts*$^+$ virus during infection of doubly seronegative vaccinees, as seen with the A/Alaska/77-*ts*-1A2 recombinant, might be anticipated with other recombinants bearing the *ts*-1A2 *ts* genes.

Determination of the Locus of the Suppressor Mutation Found in the ts-1A2 "Pseudo-revertant" Virus

A seronegative child was administered an A/Alaska/77-*ts*-1A2 recombinant that had a 37°C shutoff temperature for plaque formation. This child shed virus on days 7, 8, and 9 that produced plaques efficiently at 39°C. However, this altered virus failed to produce plaques at 40°C, a temperature at which wild type A/Alaska/77 virus produced plaques efficiently. The A/Alaska/77 *ts*$^+$ isolate was shown by segregation analysis to contain a *ts* P3 gene and a mutation on another gene that suppressed the *ts* phenotype of this *ts* P3 gene. The work this year centered on identifying the locus of the suppressor mutation. Seventeen *ts* segregants derived by mating the *ts*$^+$ isolate with a wild type virus were genotyped to determine which gene in each *ts* segregant was always derived from wild type virus. The P2 gene in each of the *ts* segregants was derived from wild type virus. In contrast, the P3 gene came from the *ts*-1A2 *ts*$^+$ isolate. At the other gene loci there was independent segregation indicating that none of these genes bore the suppressor mutation. In this manner, the locus of the suppressor mutation was identified as the P2 gene. This finding suggests that the P2 and P3 gene products interact physically because the altered P2 gene product can suppress the temperature sensitivity of the P3 gene product. Previously, the P3 and P2 genes were shown to be involved in c-RNA and v-RNA synthesis respectively, and were not thought to interact. The data from the present study suggests that they do indeed interact.

Production of a New "ts-1[E] like" Donor Virus

A new "*ts*-1[E] like" virus was produced by mating a single lesion mutant with a *ts* defect on the P3 gene with another single lesion mutant bearing a *ts* NP gene. Recombinant viruses that contained *ts* P3 and NP lesions were isolated. The original *ts*-1[E] donor virus possessed *ts* mutations on the same two genes. The single lesion donors of the ts P3 and NP genes were more stable genetically than the double-lesion ts-1[E] master strain and were chosen to produce the new ts donor virus for this reason. Three recombinant viruses were isolated, clones 20, 53 and 55, which had the following properties: (i) ts P3 and ts NP genes (by genetic analysis); (ii) 37°C to 38°C shutoff temperature for plaque formation; (iii) 10,000 fold restriction of replication in hamster lung (approximately 100-fold more restricted than the master ts-1[E] virus); (iv) no significant restriction of growth in the nasal turbinates of hamsters; (v) genetic stability in hamsters in that all isolates obtained from the lungs (12 tested) and nasal turbinates (83 tested) were ts, and (vi) replication of the mutant at 37°C (during an in vitro stress test) yielded virus that was more temperature sensitive (38°C shutoff) than the progeny of ts-1A2 recombinants subjected to the same stress.

The clone 20 virus ts NP, ts P3 mutant was mated with a wild type equine influenza A virus (Heq1 Neq1) and 2 ts equine recombinants were isolated. These studies were carried out in collaboration with Dr. Leroy Coggins at the University of Cornell School of Veterinary Medicine, Ithica, NY. The A/Cornell/64 ts recombinant clone 71 (Heq1 Neq1) had the following properties: (i) ts NP and ts P3 genes (by complementation analysis and genotyping), (ii) hemagglutinin and neuraminidase proteins of the equine parent, and (ii) growth and genetic stability characteristics in hamsters identical to the clone 20 ts parent. The other clone (clone 8) had only the ts NP gene.

The crucial question is whether the new "ts-1[E] like" viruses will have the requisite genetic stability in doubly seronegative individuals. Also, does the slightly greater stability exhibited by the new ts-1[E]-like donor and its recombinants in the stress test reflect greater genetic stability in man? The only way to answer these questions is to evaluate suitable recombinant viruses in susceptible volunteers.

Characterization of the A/Ann Arbor/6/60 Cold-Adapted Parent and Recombinant Viruses for Their Temperature-Sensitivity

We have previously demonstrated that the level of temperature sensitivity of an influenza A virus is an important determinant of attenuation for man of viruses bearing chemically induced ts mutations. The A/Ann Arbor/6/60 cold-adapted (ca) virus is ts and for this reason we sought to determine if ca derived ts mutations play a major role in the attenuation of recombinants derived from this donor. We compared the level of temperature sensitivity of the ca donor virus and recombinant H3N2 and H1N1 viruses derived from it. Parental and recombinant viruses were tested for plaque formation in MDCK monolayer cultures at permissive and restrictive temperatures. In brief, the A/Ann Arbor/6/60 ca parent virus had a 38°C shutoff temperature while the A/AA/6/60 ca recombinants had a 37°, 38°, or 39°C shutoff temperature. Each test was repeated 2 to 7 times and this permitted the demonstration of statistically significant differences in the shutoff temperature. One ca recombinant, the A/Alaska/6/77 CR29 clone 2, which received all six non-surface antigen genes from the ca parent had a 39°C shutoff temperature but was attenuated in seronegative adults and children. Chemically mutagenized ts viruses with a 39°C shutoff temperature that were studied previously retained virulence for seronegative adults. The present findings suggest that mutations other than ts mutations play a major role in attenuation of ca A/AA/6/60 recombinant viruses for man.

Development of ELISA's to Detect Antibody and Antigen

The ELISA for influenza viruses is easy to perform and appears to be more sensitive than conventional assays in the detection of an immunological response to infection. Last year we developed an ELISA using whole virus as antigen to detect infection of individuals administered an attenuated virus. We demonstrated that the difference in the quantity of antibody in acute and convalescent serum specimens was proportional to the area between the respective titration curves (O.D. versus dilution) and that this method of measuring an immune response was the single most sensitive procedure for detecting infection in individuals given an attenuated influenza A virus. We next developed an hemagglutinin specific ELISA for detecting antibody in serum and nasal wash specimens. Working with Dr. David Nelson in the Metabolism Branch of the NCI, we developed a immunoglobulin subclass-specific ELISA for measuring anti-influenza A or B hemagglutinin antibody in the IgG, IgA, IgM and IgE classes. Briefly, the ELISA for serum and nasal wash was more sensitive than the hemagglutination-inhibition assay (for serum) and the neutralization assay (for nasal wash) by about 100-fold for the former and 3-fold for the latter. These new assays which allow the detection of smaller quantities of antibody should permit us to evaluate critically the role of serum and/or nasal wash antibody in resistance to influenza A virus infection.

In addition to developing assays to detect antibody, an ELISA was developed by Dr. Berg (NIAID, NCI) and Dr. Robert Yolken, Johns Hopkins School of Medicine in collaboration with our laboratory to detect the presence of the influenza A virus hemagglutinin in secretions of individuals undergoing influenza A virus infection. The ELISA was found to be almost as sensitive as viral infectivity for detection of virus in respiratory secretions.

Development of hr Recombinants of Human Influenza A Virus and Their Evaluation in Squirrel Monkeys

Most live influenza virus vaccine strains have been produced by growth of virus in tissue culture or in a heterologous host. During this process the virus often develops mutations which adversely affect its capacity to grow in its original host, namely man. These host range (hr) mutations are difficult to identify or quantitate. Hence, there is a need to develop procedures for the detection of easily identifiable, stable hr mutations that have developed in nature over a long period of time. If successful we may be able to use such naturally occurring influenza A virus genes bearing highly stable host range (hr) mutations for attenuation of human influenza

A viruses for the purpose of immunoprophylaxis. The use of highly stable hr mutant genes represents another approach to attenuation of influenza A viruses which may yield more stable vaccine candidate strains then chemical mutagenesis. The hr mutant project will involve a collaboration with Virginia Hinshaw and Robert Webster at St. Jude's Children's Hospital in Memphis, Tenn. who will clone approximately 10 avian influenza A viruses in primary chick kidney cultures derived from specific pathogen free birds. These viruses have undergone many passages in ducks or other birds in nature and hence should contain one or more hr genes which are restricted in man and other mammalian hosts. The cloned viruses will then be evaluated for replication in ferrets, hamsters, ducks, and squirrel monkeys.

Last year we identified the squirrel monkey as a moderately permissive host for influenza A viruses belonging to H1N1 and H3N2 subtypes. This animal model system was developed for use in evaluating potential host range mutants of influenza A virus in a non-human primate before initiating studies of such mutants or their recombinants in man. In order to study hr mutants comprehensively it is necessary to evaluate the level of virus replication in the lower as well as upper respiratory tract so that restriction produced by hr mutations can be assessed throughout the entire respiratory tract. In previous studies only the latter site was sampled. This year we determined whether tracheal lavage could be repeatedly performed in order to sample the lower respiratory tract of squirrel monkeys infected with an H3N2 wild type influenza A virus. This manipulation did not induce disease but did yield virus in high titer from bronchial lavage specimens. We are now in a position to determine the level of replication of various avian influenza A viruses in both the upper and lower portions of the respiratory tract of squirrel monkeys. Furthermore, avian viruses that grow significantly less well than human influenza A viruses throughout the respiratory tract can be identified. Avian influenza viruses that exhibit a 100 to 1000-fold reduction of replication in both the upper and lower respiratory tract will be evaluated further through the study of appropriate recombinants to identify the genetic determinants of this restriction. Recombinants bearing the "host range" genes of an avian virus and the surface glycoproteins of a human virus will be produced and evaluated as candidate donor viruses in the squirrel monkey model. Subsequently, promising recombinants will be evaluated in man

Publications

Massicot,J.G., Murphy, B.R., Thierry, F., Markoff, L., Huang, K., and Chanock, R.M.: Temperature-sensitive mutants of influenza virus: Identification of the loci of the 2 ts lesions in the Udorn-ts-1A2 donor virus and the correlation of the presence of these 2 ts lesions with a predictable level of attenuation. Virology 101: 242-249, 1980.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00026-13 LID

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
LABORATORY AND EPIDEMIOLOGIC STUDIES OF VIRAL HEPATITIS AGENTS

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	R. Johnson	Research Associate	LID,NIAID
	J. Ticehurst	Research Associate	LID,NIAID
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	M. Canese	Visiting Scientist	LID,NIAID
	M. Rizzetto	Visiting Scientist	LID,NIAID
	I. Gust	Visiting Scientist	LID,NIAID
	Y. Shimizu	Visiting Associate	LID,NIAID
	B. Hansson	Visiting Fellow	LID,NIAID
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Other: P. Holland, H. Alter (CC, Blood Bank, NIH), J.L. Gerin (Georgetown University), W. London (NINCDS), D. Sly (Meloy Labs)

LAB/BRANCH
LABORATORY OF INFECTIOUS DISEASES

SECTION
HEPATITIS VIRUSES SECTION

INSTITUTE AND LOCATION
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES, BETHESDA, MARYLAND

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
12.5	9.5	1.5

CHECK APPROPRIATE BOX(ES)
☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project consists of continuing studies of the chemistry, structure, epidemiology, immunology and pathology of human and related hepatitis viruses. The goal of such studies is the control of human viral hepatitis by application of the most appropriate methods, including active and passive immunization, chemotherapy and interdiction of spread of the viruses. Progress: Hepatitis A virus has been isolated directly from the stool of a patient into primary African green monkey kidney cell culture, a substrate suitable for vaccine development. The RNA genome of hepatitis A virus is being cloned in *E. coli*. Modifications in hepatitis B subunit vaccine have resulted in a highly immunogenic and safe preparation that produces a seroconversion in approximately 60% of vaccinees within two weeks of vaccination and in 95% following completion of the vaccination schedule. Non-A, non-B hepatitis agents have been transmitted to chimpanzees and, probably, marmosets, and ultrastructural changes that appear to be specific for non-A, non-B hepatitis have been identified. Evidence has been obtained for the existence of an epidemic form of non-A, non-B hepatitis in India. Attempts to transmit the virus to chimpanzees are currently in progress.

Hepatitis A Virus

Hepatitis A virus (HAV) resembles most closely the picornaviruses: It is a 27 nm particle with cubic symmetry that consists of four polypeptides with molecular weights similar to those of the picornaviruses and a single stranded linear RNA genome. However, it is more heat resistent than the picornaviruses, can be found at a wider range of buoyant densities and may have a somewhat smaller genome.

Isolation of HAV in Tissue Culture - HAV was recently isolated in tissue culture for the first time by Provost. Successful isolation followed 31 serial passages in marmoset monkeys before recovery in fetal rhesus kidney diploid cell culture. Still more recently Frosner recovered HAV directly from feces in Alexander hepatoma cells, a transformed human cell line that probably contains the complete hepatitis B virus genome. We have attempted to isolate HAV directly from clinical materials into a cell line suitable for vaccine development. These studies, under the direction of Dr. Richard Daemer, have utilized three different strains of HAV: The MS-1 strain studied by Krugman at Willowbrook, the SD-11 strain recovered by Dienstag from a Naval recruit in San Diego and the HM-175 strain supplied by Dr. Gust from Australia. When available, HAV-containing serum samples and fecal samples were both employed for isolation attempts. Immunofluorescence and, to a lesser extent, radioimmunoassay were used as indicators of virus multiplication. All three strains of HAV were serially passaged in marmosets up to 9 times, and marmoset-passaged virus was compared, at various passage levels, with HAV-containing clinical materials in isolation attempts. Although partially successful results were obtained with all three strains, consistently better results were obtained with the HM-175 strain, and subsequent studies were carried out only with this strain. Both marmoset passaged (pass 6) HAV and HAV in a fecal suspension were successfully isolated in primary African green monkey kidney (AGMK) cell culture. Multiplication of the virus at early passage levels was slow, requiring 8 or more weeks to achieve moderate to intense fluorescence in the cultures. RIA tests revealed that all of the detectable antigen was cell-associated. For this reason subsequent passage of the virus was carried out by passage of live cells. HAV is currently in 7th passage in primary AGMK, and the duration of each passage has shortened from approximately 8 weeks to 3-4 weeks. Virus isolated directly from feces is growing as well as marmoset-passaged virus. In contrast to the results obtained with the primary AGMK, similar attempts to isolate the HM-175 strain of HAV in WI-38 and MRC-5 cell lines were totally negative, even after 5 serial blind passages. We are currently attempting to adapt HAV grown in primary AGMK to growth in diploid cultures.

Cloning of HAV - Because HAV is difficult to grow in tissue culture and because a number of questions remain about it molecular virology and taxonomy, we have attempted to clone the HAV genome in *E. coli*. If successful such a cloned genome would be useful in studies of viral nucleic acid synthesis. Dr. John Ticehurst has purified HAV from infected marmoset livers and extracted viral RNA. Using a series of enzymatic steps he has prepared complementary DNA and from this has prepared double-stranded DNA consisting of strands complementary to positive and negative strands of viral RNA. This double-stranded DNA has been inserted into an appropriate plasmid and has been replicated in *E. coli.* At least one clone of plasmid contains DNA that hybridizes with the original RNA preparation. Experiments are currently in progress to confirm that this clone, is, indeed, derived from HAV RNA.

Preparation of Monospecific (hybridoma) antibody to HAV - Virtually all antisera used for the diagnosis and characterization of HAV infection are convalescent sera obtained from marmosets, chimpanzees or man. In order to prepare more specific reagents Dr. Vin McAuliffe has attempted to prepare hybridoma antibodies from mice hyperimmunized with HAV partially purified from marmoset liver. Several hybridoma clones that synthesize antibody to HAV have been detected and are currently being purified and further characterized.

Radioimmunoassay for detecting IgM-class anti-HAV - Antibody to HAV is almost always present by the time clinical signs and symptoms appear, thereby making diagnosis of an acute infection difficult. However, tests that would permit detection of the IgM class of anti-HAV could be used for diagnosis of recent HAV infection with a single acute phase serum. Dr. Bengt Hansson developed a solid-phase radioimmunoassay during his stay in the Hepatitis Viruses Section under the Fogarty Visiting Program. The test is quite sensitive, detects antibody for approximately three months after onset of illness and requires only a small amount of serum.

Hepatitis B Virus

Hepatitis B virus (HBV) is a unique, 42nm virus of complex structure that contains a double-stranded circular DNA with a single stranded gap spanning 20-50% of the genome. Recently three other viruses with similar characteristics have been discovered: The woodchuck hepatitis virus, (WHV), the Beachey ground squirrel virus and the duck hepatitis virus. Undoubtedly other members of this group will be discovered in other species. HBV is the cause of an enormous amount of morbidity and mortality, accounting for 30-50% of clinical hepatitis diagnosed in the U.S. However, it is in Asia and Africa where the virus causes the greatest damage, resulting in chronic infection of 5-10% of the population and leading to chronic hepatitis and hepatic cell carcinoma in a significant proportion of these individuals. For these reasons control of HBV is an important public health goal.

HBV Vaccine - Work has continued on the development and characterization of hepatitis B vaccine. Modifications made in the vaccine formulation have resulted in a highly immunogenic preparation, capable of stimulating antibody in a majority of recipients receiving as little as 5 ug of vaccine and producing a rapid and brisk antibody response: 60-80% of vaccinees develop antibody within one month of vaccination and 95% seroconvert by completion of the vaccination series. The NIAID vaccine produces antibody in approximately 2-3 times as many recipients by one month after vaccination as other vaccines currently undergoing testing. For this reason we believe that the NIAID vaccine will be useful in those situations where rapid antibody response is essential, such as interruption of the maternal-fetal transmission of HBV that is common in Asia. Plans are underway to carry out an efficacy trial of the NIAID hepatitis B vaccine in such a setting.

Woodchuck Hepatitis Virus - The similarities between HBV and WHV, coupled with the tendency for each virus to lead to chronic hepatitis and hepatic cell carcinoma in their respective hosts, makes WHV and its host the woodchuck, a particularly interesting animal model system. In collaboration with Dr. Gerin and Dr. Popper, we have been studying the virology and pathology of WHV-associated hepatitis and hepatoma in woodchucks. Sensitive serologic tests are being developed for the WHV antigens and antibodies and attempts to transmit the virus to both woodchucks and primates are in progress. Dr. Popper is characterizing the histopathology of acute and chronic hepatitis and hepatic cell carcinoma in animals that were naturally infected. These studies should not only better clarify the role of hepatitis viruses in the etiology of hepatic cell carcinoma, but may provide a means of predicting whether hepatitis vaccines will prevent hepatic cell carcinoma.

Non-A, Non-B Hepatitis Agents

As serologic tests for HAV and HBV infection are more widely applied it is becoming apparent that non-A, non-B hepatitis agents are widely distributed and associated with a significant proportion of clinical hepatitis cases, perhaps 25% of all clinical cases and as much as 90% of post-transfusion hepatitis. A number of such agents have been successfully transmitted to chimpanzees, but despite intensive efforts, an antigen-antibody system unequivocally specific for non-A, non-B infections has not been detected. An exception is delta antigen, an antigen associated with a unique non-A, non-B agent.

Delta Antigen- In 1977, Mario Rizzetto discovered a new antigen in patients with chronic type B hepatitis. This antigen, detected by fluorescence, was found in hepatocyte nuclei of patients with chronic type B hepatitis. Its distinctness from hepatitis B core antigen was demonstrated. Extensive studies with delta antigen were carried out by Dr. Rizzetto as a Fogarty Visiting Scientist in collaborative studies with Dr. Gerin's laboratory and the Hepatitis Viruses Section. Delta was found to be associated with a transmissible agent that required HBV synthesis for its own synthesis and that supressed the synthesis of HBV markers. Thus, it had the characteristics of a defective interfering agent. Delta could be detected by immunoflourescence in the liver of chimpanzees experimentally infected with the agent but could not be detected in the serum until centrifugal pellets of the serum samples were treated with detergent. Thus, delta appeared to be enclosed within a particle. When samples of chimpanzee plasma containing delta were subjected to centrifugal separations, delta copurified with 35-37nm particles that resembled large HBsAg particles. Furthermore, these particles were coaggregated with the 22nm HBsAg particles by anti-HBs. Thus, delta was an internal component of particles incapsidated by HBsAg. When such particles were extracted for nucleic acid, a low molecular weight (approx. 500,000 daltons) piece of RNA was detected. This RNA also appeared to be enclosed, with delta, inside a coat of HBsAg. Delta could be readily transmitted to chimpanzees either acutely or chronically infected with HBV but could not be transmitted to HBV-immune chimps. However, electron micrographic changes similar to those seen during acute non-A, non-B hepatitis infection (see below) have been detected in such chimpanzees, suggesting that delta may share some characteristics in common with other non-A, non-B agents.

Dr. Rizzetto developed a radioimmunoassay for delta and anti-delta and performed seroepidemiologic studies. He found that delta had a worldwide distribution but that it was highly endemic in Italy, especially in southern Italy, where it was assocaited with 50% of acute HBV infections. In Europe and the U.S. evidence of infection with delta was found most frequently in those extensively exposed to blood and blood products (hemophiliacs, illicit drug users, etc.). In summary, delta appears to be a unique transmissible agent with characteristics unlike those of any previously described self-replicating agent.

"Classical" Non-A, Non-B Agents - The epidemiology of the non-A, non-B agents resembles that of HBV quite closely: the agents are primarily transmitted by blood and blood products and lead to chronic infection in a high proportion of patients. Until recently (see below) the non-A, non-B agents were not associated with fecal-oral transmission or common-source outbreaks of hepatitis. Although satisfactory serologic tests have not been developed, Dr. Yohko Shimizu, while a Fogarty Fellow, did detect unique electronmicrographic changes in hepatocytes of chimpanzees experimentally infected with non-A, non-B agents. Two types of changes were observed, cytoplasmic changes associated with fusion of membranes of the endoplasmic reticulum into peculiar cylindrical structures and nuclear changes characterized by pycnosis, heterogeneous staining and, in a small proportion of cells, clusters of particles. In initial experiments, each of these electronmicrographic changes was seen independently, suggesting that they were caused by two different agents. Both changes have subsequently been found in chimpanzees inoculated with the same inoculum, and it is not yet clear whether the changes represent different stages of infection or whether the inocula contain more than one agent. Attempts to sort this out are currently in progress.

Dr. Stephen Feinstone has continued studies on the transmission of non-A, non-B agents to primates and has completed the infectivity titration of two strains, one that produces cytoplasmic changes and one that produces predominantly nuclear changes. The former has an infectivity titer of only approx. Ten chimpanzee infective doses (CID) per ml; the latter has an infectivity titer of approx. 10^7 to 10^8 CID per ml. Such titered virus suspensions will now be used to begin the characterization of these agents.

In addition, Dr. Feinstone has successfully transmitted non-A, non-B agents of both types to marmosets. The nuclear changes were reproduced in marmosets, but the cytoplasmic changes have not been seen to date.

In collaborative studies with Dr. Harvey Alter and Dr. J. Hoofnagle, liver biopsies from patients with chronic non-A, non-B hepatitis are being examined by electronmicroscopy by Dr. Hans Dienes. In several such biopsies he has found the peculiar nuclear particles that have been seen in chimpanzee and marmoset livers of animals infected with one type of non-A, non-B agent. Thus, certain electronmicrographic changes appear to be found consistently in various species infected with non-A, non-B agents.

Indian Hepatitis - In collaborative studies with Dr. Khorshed Pavri, Mrs. Doris Wong has found that two outbreaks of epidemic water-borne hepatitis and one cluster of cases of endemic hepatitis in India could not be serologically related to infection with HAV or HBV. Thus, these cases, which resembled type A hepatitis in many respects, appeared to be caused by non-A, non-B agents. It is not clear whether these agents represent one or more previously unrecognized serotypes of HAV or whether they are entirely new agents. Attempts to transmit them to chimpanzees and marmosets are currently in progress.

Other Studies

Anti-DNA Antibodies - Villarejos and others reported that patients with hepatitis of whatever etiology developed antibodies to single-stranded DNA during the acute phase of infection. Since detection of such antibody would be a useful tool for the study of pathogenesis of hepatitis as well as possibly a method of diagnosis for inapparent non-A, non-B hepatitis, we to attempted to confirm this finding. Dr. Ian Gust, during his sabbatical stay in the Hepatitis Viruses Section, established several published assays for anti-DNA antibodies and compared results obtained from patients with other immune diseases to those in patients and experimentally infected chimpanzees with various types of viral hepatitis. He could not confirm the report that anti-single stranded DNA antibodies developed routinely during acute viral hepatitis. Coded samples of serum from naturally infected humans and experimentally infected chimpanzees were distributed to the laboratories claiming success in the detection of such antibodies. The laboratories could not successfully break the code or agree between themselves even though they had published the original reports as collaborative studies.

Histopathology of Viral Hepatitis in Chimpanzees - In collaboration with Dr. Hans Popper, we have examined the similarities and dissimilarities of histologic changes occurring in the liver of chimpanzees experimentally infected with HAV, HBV or non-A, non-B agents. All samples were evaluated by Dr. Popper under code. Subtle but distinct differences in histopathologic lesions caused by the three viruses were detected.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00027-13 LID

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
BASIC STUDIES OF MYCOPLASMAS

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Joseph G. Tully, Ph.D.	LID, NIAID	Head, Mycoplasma Section
Other:	David L. Rose	LID, NIAID	Res. Microbiologist

COOPERATING UNITS (if any) State Univ. N.Y., Stony Brook, N.Y.; USDA Beltsville, Md.; Univ. Maryland, Baltimore; Univ. Bordeaux, France, Connecticut Exp. Station, New Haven, Conn.; Cornell Univ. New York, Rocky Mountain Laboratory, NIAID

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INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

These efforts cover both basic and applied aspects of mycoplasmas and related wall-free prokaryotes, including their neurotoxins, antigens, and other biological factors involved in virulence, their immunological interrelationships, and their possible role in human disease or diseases of uncertain etiology. Current projects concern the characterization, pathogenicity, and serological properties of an expanding group of helical mycoplasmas (spiroplasmas) being isolated from plants and a variety of insects (especially ticks). Seven new spiroplasma strains have been isolated from Ixodes pacificus ticks collected in Oregon. These organisms appear to represent a completely new, and serologically-distinct, group of spiroplasmas. Serological analysis of most known spiroplasmas has shown at least six distinct serogroups, as based upon the spiroplasma metabolism inhibition test developed in this laboratory. This information will assist sero-epidemiological studies on human sera with these organisms. Experimental infections of suckling rats with a pathogenic spiroplasma has failed to show a clear inflammatory response in brain tissue, despite the fact that the animals have central nervous system symptoms and as many as 10^5 infectious units/gram of brain tissue. However, spongiform changes were seen.

PROJECT DESCRIPTION

Studies on the biology and pathogenicity of helical mycoplasmas (spiroplasmas) have continued, with current efforts directed to (1) their biological and serological characterization, (2) development of appropriate models to study pathogenicity, (3) distribution in hosts in contact with man, and (4) evaluation of various techniques (serology, isolation, etc.) to assess the possible role of spiroplasmas in acute and chronic human disease. Further information on the epidemiology of Mycoplasma pneumoniae infections was derived from a retrospective study on throat cultures obtained from military recruits participating in an earlier (1974) vaccine field trial.

(A) Spiroplasmas in new hosts

Although spiroplasmas pathogenic for vertebrates have been recovered from rabbit ticks, little information is available on the occurrence of these organisms in other ticks, particularly in ticks with a more intimate association to man. Over 80 tick pools, representing primarily three species of Ixodes and four species of Dermocentor, have been cultured on SP-4 spiroplasma medium. The largest number of ticks examined (30 pools totaling 261 ticks) have been those of Ixodes pacificus collected in Oregon from December, 1979 through May, 1980. Seven of these pools have yielded spiroplasmas and the new isolates are now being carried in continuous culture and undergoing purification procedures. Preliminary evidence would suggest that these spiroplasmas represent at least one new species of serogroup. These new spiroplasmas provide several new antigens to use in serological tests on human sera. More extensive efforts are underway to culture spiroplasmas from Ixodes damini collected in Connecticut. This latter species of tick has been associated with outbreaks of a clinical syndrome (Lyme arthritis) in humans which has yet to be identified with a specific microbial agent. (Yunker, RML: NIAID; Anderson, Connecticut)

(B) Serological Testing of Spiroplasmas

The metabolism inhibition test (adapted in this laboratory to spiroplasma serology) has been applied to a comprehensive serological analysis of all known spiroplasmas. The results established five distinct serological groups of these agents, including a new group consisting of 5 strains recovered from insects and plants. The serogrouping observed has been confirmed by DNA hybridization tests and differences in the values of the G + C ratio of the DNA of these organisms. A proposed new classification scheme of spiroplasmas, based upon our serological data and collaborative work on their genetic properties, has been offered. The results of these studies have clarified the interrelationships of spiroplasmas and provided data necessary to expand sero-epidemiological studies on spiroplasma antibody in man and other vertebrates. (Williamson, SUNY; Whitcomb, USDA: Bove, Bordeaux, France)

(C) Pathogenicity of spiroplasmas

Virulence testing of a large collection of spiroplasmas in embryonated eggs and suckling rats has suggested the presence of some unique pathogenicity markers in these organisms. A number of spiroplasmas recovered from plants and insects induced central nervous system disease in suckling rats and were lethal to the chick embryo. These virulent spiroplasmas, in addition to the highly virulent suckling mouse cataract spiroplasma (SMCA), provide useful models to understand virulence mechanisms in these organisms. Studies directed to correlation of the neuropathological response with numbers of spiroplasmas in the rat model have shown that animals with as many as 10^5 spiroplasmas per gram of brain tissue and obvious central nervous system symptoms did not have an inflammatory response or other pathological responses in the brain. A spongiform encephalopathy was, however, apparent. Electron microscopic and immunofluorescence techniques are now being applied to the study of this brain material, with the view of identifying organisms and their specific tissue localization. This type of study is being carried out in relationship to a histopathological and cultural study on human brain material from Creutzfeldt-Jakob (CJ) disease. Two cases of CJ disease have been observed (Bastian, 1979) to have some helical filaments similar in size and shape to spiroplasmas. The experimental model of SMCA in the suckling rat is specifically directed to techniques which may identify spiroplasmas in CJ disease and in other chronic brain diseases in man. (Bastian, Univ. Maryland)

(D) *Mycoplasma pneumoniae* infections

The SP-4 medium developed in this laboratory for isolation and cultivation of spiroplasmas was found to increase the efficiency of isolation of *M. pneumoniae* from human throat specimens. Two hundred frozen throat specimens from military recruits participating in a 1974 vaccine field trial were cultured on SP-4 medium. These specimens had been cultured earlier on conventional mycoplasma medium and found to be negative for *M. pneumoniae*. Thirty-four percent of specimens were positive for *M. pneumoniae* on SP-4 medium. More recently, 200 additional specimens were cultured with similar results (34% positive for *M. pneumoniae*). These findings are now being correlated with a number of serological tests on recruit sera, representing those with positive isolations, to determine whether the increased isolation rate will alter the previously established efficacy (65%) of the inactivated *M. pneumoniae* vaccine. The most important observation of this retrospective study is that 10-20% of the recruits with positive isolations of *M. pneumoniae* did not possess complement-fixation antibody to the organism or have evidence of respiratory disease. In some cases, follow-up serum samples obtained two to three months after the positive isolation of the organism were still negative for CF antibody. (Senterfit, Cornell Univ.)

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 000-28-22 LID

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
STUDY OF RESPIRATORY VIRUSES AND MYCOPLASMAS IN VOLUNTEERS

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Brian R. Murphy,	Medical Officer	LID, NIAID
	Robert M. Chanock	Chief	LID, NIAID
	Mark D. Tolpin	Medical Officer	LID, NIAID

COOPERATING UNITS (if any) Flow Laboratories, Rockville, Md.; University of Maryland School of Medicine, Baltimore, Maryland; University of Rochester School of Medicine, Rochester, NY; Children's Hospital National Medical Center, Washington, D.C.; Center for Disease Control, Atlanta, Georgia

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Three A/Alaska/77 ca recombinants of A/Ann Arbor/6/60 ca donor virus evaluated in man for level of attenuation. Each of the three were similarly and satisfactorily attenuated despite having different genotypes. These observations suggest that M and NS genes of the ca donor virus are not major attenuating genes. A ts-1A2 "revertant" containing suppressor and other mutations leading to loss of ts phenotype was virulent in adult volunteers. This finding identified the ts genes of the ts-1A2 recombinants as the genes responsible for attenuation. Recombinants possessing the two ts-1A2 ts genes may not be sufficiently stable genetically and loss of the ts property could have potential epidemiological significance.

Project Description

Evaluation of Cold-Adapted Recombinants in Adults - The influenza A/Ann Arbor/6/60 cold-adapted (ca) virus is being evaluated as a donor of attenuating genes to new variants of influenza virus. Ca recombinant viruses which were derived from the A/Ann Arbor/6/60 ca virus have been attenuated for animals and man. However, the A/AA/6/60 genes that confer attenuation have not as yet been identified with certainty. It is important to determine which A/AA/6/60 ca genes confer attenuation and to identify these attenuating genes in future ca recombinant viruses derived from this parent. To this end collaborative studies with Dr. Maassab at the University of Michigan School of Public Health and Dr. Kendal at the Center for Disease Control, Atlanta, have continued. Dr. Maassab prepares the ca recombinants, Dr. Kendal genotypes the recombinants, and our laboratory collaborates with scientists at the University of Maryland School of Medicine and Rochester University School of Medicine to evaluate the ca recombinants in volunteers. Studies were carried out this year with three cloned A/Alaska/77 (H3N2) ca recombinants. One recombinant possessed all six transferable A/AA/6/60 genes (i.e., genes other than those coding for the surface glycoprotein hemagglutinin and neuraminidase antigens) and the other two received only 5 of the 6 transferable genes with a wild-type gene at the M or NS locus. Initially, the ca recombinant bearing all six transferable A/AA/6/60 genes ("6" ca gene recombinant) was found to be satisfactorily attenuated and antigenic in seronegative adult volunteers. However, only 18 of 24 volunteers were infected. Next, the ca recombinants bearing only five of the six transferable genes were evaluated. These "5" ca gene recombinants were identical to the recombinant bearing all six transferable genes except that they had a wild type gene substituted at the NS or M locus. If the "M" or "NS" gene of the ca donor strain is a major determinant of attenuation, loss of such an attenuating gene should be associated with increased virulence. Virulence of the "5" ca gene recombinants was assessed by determining the duration and magnitude of virus shedding and the clinical response of seronegative adult volunteers to these viruses. The "5" and "6" ca gene A/Alaska/77 recombinants were each satisfactorily attenuated and were indistinguishable from each other as regards virulence. Each of the recombinants was shed in significantly lower quantity and for a shorter duration than was wild type A/Alaska/77 virus. Only two mild illnesses were observed among 46 volunteers infected with the ca recombinants compared to a 50% illness frequency among volunteers infected with wild type virus. The two illnesses induced by the ca recombinant, were of lesser magnitude and of shorter duration than the illnesses caused by wild type virus. These observations suggest that the M and NS genes are not significant determinants of attenution of ca recombinant viruses. Studies performed previously suggested that RNA-2 gene of the A/AA/6/60 ca virus was not a major attenuating gene. Considered together,these results suggest that RNA-1, RNA-3 and the NP genes, which are present in all ca recombinants that have the ts and ca properties, are most likely the major attenuating genes of the A/Ann Arbor/6/60 ca donor virus. However, one cannot rule out the possibility that one or more wild type genes present in a ca recombinant undergo mutation during recombination and cloning which are performed at 25°C, a temprature undoubtedly highly selective for spontaneous mutation.

Future studies with the *ca* recombinant viruses will be directed at identifying the genetic basis for their attenuation. To determine if the *ts* phenotype contributes to the attenuation of these viruses for man, *ts*$^+$ *ca* recombinant viruses will be generated *in vitro* and evaluated for their level of attenuation in man. In addition, *ca* recombinant viruses bearing various mixtures of *ca* and wild type genes at the non-glycoprotein loci will be evaluated for their level of attenuation in man to determine which *ca* genes bear the attenuating mutations.

Studies with a "Revertant" Virus Derived From the A/Alaska/77-ts-1A2 Virus - The A/Alaska/77-*ts*-1A2 virus was given to a doubly-seronegative child and illness was not observed, but virus with an altered temperature-sensitive phenotype was isolated. The A/Alaska/72-*ts*-1A2 virus that was recovered on days 7, 8, and 9 was clearly less *ts* (40°C shutoff temperature for plaque formation *in vitro*) than the input *ts*-1A2 virus which had a 37°C shutoff temperature. However, the altered *ts*-1A2 virus (*ts*$^+$) was more *ts* than A/Alaska/77 wild type virus which had a shutoff temperature of greater than 40°C. Subsequently, it was demonstrated that the *ts*$^+$ virus recovered from the child had undergone several new mutations during growth *in* vivo:. 1) The *ts*$^+$ virus possessed a new mutation on the P2 gene that suppressed the *ts* phenotype of the *ts* P3 gene. 2) The *ts* P3 gene migrated more slowly on polyacrylamide gel than the parental P3 gene present in the *ts*-1A2 virus administered to the vaccinee. 3) The *ts* P3 gene was less *ts* than its parental gene in the *ts*-1A2 virus indicating this gene underwent mutation. 4) The *ts* P1 gene lost its *ts* phenotype.

It was important to assess the clinical significance of these mutations to determine if there was a restoration of virulence associated with those genetic changes. The *ts*$^+$ virus recovered on day 9 was cloned at permissive temperature (34°C) and then restrictive (39°C) temperature. Subsequently the *ts*$^+$ virus was administered to seronegative adult volunteers at a dose of $10^{4.0}$ or $10^{5.0}$ $TCID_{50}$. The quantity of virus shed, the pattern of virus shedding and the illnesses observed were comparable to those seen in volunteers infected with wild type virus. These results suggested that the *ts* phenotype of the *ts*-1A2 recombinant was a major determinant of attenuation. The high titer of *ts*$^+$ virus shed by the vaccinee (10^5 $TCID_{50}$ per ml of nasal wash fluid) suggests that restoration of the *ts*$^+$ phenotype might lead to transmission of altered virus to suceptible contacts under the appropriate conditions of exposure. Genetic instability leading to restoration of the *ts*$^+$ phenotype and possible spread of *ts*$^+$ virus to contacts precludes widespread use of *ts*-1A2 recombinants for immunoprophylaxis despite the fact that these recombinants are satisfactorily attenuated for doubly seronegative (highly susceptible) volunteers.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00175-03 LID

PERIOD COVERED October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

LABORATORY STUDIES OF PARAMYXOVIRUSES AND RESPIRATORY SYNCYTIAL VIRUS

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

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LAB/BRANCH
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SECTION
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TOTAL MANYEARS: 5.5	PROFESSIONAL: 2.5	OTHER: 3.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Two mutants were evaluated in seronegative chimpanzees. Both mutants retained some virulence for the upper respiratory tract suggesting that they were not sufficiently attenuated for use in susceptible infants and young children. The phenomenon of immunosuppression was studied in greater detail by administering pooled cotton rat RS virus antisera intraperitoneally (IP) to seronegative animals which were than immunized IM with live wild-type virus. Those animals which were given antisera were unable to respond to the IM vaccine, whereas the animals which received pooled control sera (free of RS virus antibody) prior to vaccination were successfully immunized. This demonstrated that serum antibody or a concomitant serum immune factor, could produce immunosuppression. The immunosuppressive effect seen in IM vaccination was not seen during IN vaccination, and thus topically applied RS virus vaccines may confer effective immunity independent of antibody in serum at the time of vaccination.

PHS-6040
(Rev. 10-76)

Project Description

This year in the respiratory syncytial (RS) virus program emphasis was given to 5 areas of research: 1) evaluation of ts mutants and cold-adapted mutants that appeared to be promising vaccine candidates; 2) immunological studies of RS virus infection in experimental animals; 3) assessment of the structural alterations and antigenic patterns of fatal human RS virus disease; 4) influence of selected oligopeptides and Ribavirin in in vitro and in vivo RS virus replication; 5) demonstration and characterization of the proteins of RS virus.

1) Evaluation of Vaccine Candidates

Background - RS virus is the major etiologic agent of bronchiolitis and pneumonia of early life, and the need for effective immunoprophylaxis has been clearly established in studies performed throughout the world. This virus has a non-segmented genome and does not undergo genetic reassortment. Initially a series of 7 ts mutants of RS virus were produced by chemical mutagenesis with the expectation that their temperature sensitivity would restrict growth in the target organ, the lungs. Additional cold-adapted strains were derived as possible vaccine candidates.

Primate studies

Cold adapted (ca) RS virus - Clone 14, lot #1075 of a cold-adapted (ca) RS virus prepared by the Sandoz Forschungsinstitut was inoculated intranasally into two seronegative chimpanzees. The viral growth pattern as well as the severity and duration of rhinorrhea could not be distinguished from that observed with wild type RS virus, indicating that this ca virus was as virulent as unmanipulated wild type RS virus. We were unable to confirm that the ca virus from Sandoz was a ts mutant. In our tests the ca virus did not exhibit a ts phnotype.

Evaluation in chimpanzees of the ts-1 NG-16 mutant grown in MRC-5 human diploid cell culture - The ts-1 NG-16 virus suspension grown in bovine embryonic kidney (BEK) cell culture was found to be contaminated with bovine mucosal diarrhea (BVD) virus by Dr. J. Stott. In collaborative studies it was established that BVD virus did not infect man. Nonetheless it was desirable to remove this contaminant from the ts-1 NG-16 virus passage material and prepare a new suspension of the RS virus mutant for evaluation in primates and possibly man. The BVD virus was removed by exposing the BEK grown RS virus to BVD antiserum and then growing it in MRC-5 human diploid cells which are refractory to BVD virus. This was done successfully. Comparison of the MRC-5 culture grown ts-1 NG-16 mutant with the parental virus grown in BEK cells failed to demonstrate an alteration in ts phenotype.

The MRC-5 culture grown ts-1 NG-16 mutant was evaluated in 2 seronegative chimpanzees. The chimpanzees developed definite rhinorrhea which started on the 6th day post-infection and lasted for 10 days. The pattern of virus shedding and the quantity of virus shed did not differ from that observed during infection with wild type virus. These findings, not unlike those observed with the ts-1 mutant (the progenitor of the ts-1 NG-16 mutant), suggest that ts-1 NG-16 mutant may retain some virulence for the upper respiratory tract of highly susceptible infants and young children.

Bronchoscopic evaluation of the lower respiratory tract of chimpanzees - Since the pathologic process which causes serious human illness is confined to the lower respiratory tract, evaluation of this area in experimentally infected animals is required in order to assess the safety and efficacy of candidate vaccine viruses. We found that bronchoscopy, bronchial lavage, bronchial brushing, and transbronchial biopsy can be performed safely and without injury to the chimpanzee. Future vaccine trials will include these additional assessments of the lower respiratory tract. Regretably these procedures cannot be performed on the more readily available owl monkeys (Aotis sp.) because of their small size.

Studies in Cotton Rats

ts-1 NG-1 and ts-1 NG-16 mutants - Previous studies in owl monkeys showed that the ts-1 NG-1 and ts-1 NG-16 mutants were as infectious for the upper respiratory tract and as antigenic as wild type virus, yet these mutants were significantly attenuated. Studies conducted this year in the cotton rat extended these earlier findings. Both mutants were markedly restricted in their ability to replicate in cotton rat tissues, particularly the lungs. The ts1 NG-1 mutant was more restricted in its growth in the lungs than the mutant (ts-1) from which it was derived by additional mutagenesis with nitrosoguanidine (NG). In contrast, the degree of restriction in growth exhibited by the ts-1 NG-16 mutant was equivalent to that seen with its parental mutant (ts-1).

Forty-one isolates of ts-1 NG-1 and ts-1 NG-16 from cotton rat nasal and pulmonary tissues were evaluated for genetic stability by examining the ability of the isolates to produce plaques in tissue culture at restrictive temperatures. Forty of the 41 isolates exhibited the same temperature sensitivity as the input virus, while one isolate, obtained from the lungs of an animal infected with ts-1 NG-16, showed a partial shift toward wild-type phenotype, but complete reversion was not observed. Both viruses, therefore, appear to be genetically stable in vivo.

Antibody production in cotton rats infected with either ts-1 NG-1 or ts-1 NG-16 was low, with some animals failing to develop detectable serum neutralizing antibody. In spite of this poor immunologic response, both mutants induced nearly complete or complete resistance to subsequent challenge by wild-type virus. It appears that the properties of attenuation, genetic stability in vivo, and protective efficacy of ts-1 NG-1 and ts-1 NG-16 make both mutants promising vaccine candidates.

2) Immunosuppressive effect of serum antibody on response of cotton rats to live RS virus administered intramuscularly (IM)

Since an RS virus vaccine is most urgently needed during the first few months of life, the issue of immunosuppression by maternally derived serum antibody represents a potential obstacle to successful immunization in this age group. Work reported last year showed that cotton rats born to mothers which had previously been infected with RS virus were refractory to intramuscular (IM) vaccination with live wild-type virus. In contrast, animals born to mothers without prior exposure to RS virus were successfully vaccinated by the IM route. Although specific immune factors were responsible for the immunosuppressive effect, the nature of these factors, and the amount necessary to block immunization were not determined. We examined the phenomenon of immunosuppression in greater detail by administering pooled cotton rat RS virus antisera intraperitoneally (IP) to seronegative animals which were than immunized IM with live wild-type virus. Those animals which were given antisera were unable to respond to the IM vaccine, whereas the animals which received pooled control sera (free of RS virus antibody) prior to vaccination were successfully immunized. This demonstrated that serum antibody or a concomitant serum immune factor, could produce immunosuppression.

A second experiment, designed to determine the dilution endpoint of serum blocking factors required for immunosuppression, showed that pooled antisera diluted 1:256 was sufficient to completely block IM vaccination, although this amount of antisera did not yield detectable neutralizing antibody in the serum of recipient rats. This suggests that a live virus vaccine administered IM may not be useful in individuals most in need of immunoprophylaxis, since the greatest impact of RS virus occurs during the first six months of life when most infants possess maternally derived, serum neutralizing antibody for RS virus.

The immunosuppressive effect of antibody on parenteral RS virus vaccination raises the possibility of a similar effect during immunization with a live attenuated virus administered into the respiratory tract. To study this possibility, we inoculated seronegative cotton rats IP with the pooled antisera described previously, then administered live, attenuated RS virus (*ts*-1 NG-16) intranasally (IN) the following day. Following challenge with wild type virus (three weeks later) all the animals were shown to be resistant to challenge (IN) with wild type virus, whether they had received immune or control (antibody-free) sera prior to vaccination. It would appear that the immunosuppressive effect seen in IM vaccination is *not* seen during IN vaccination, and that topically applied RS virus vaccines may confer effective immunity independent of antibody in serum at the time of vaccination.

3) Assessment of Structural Alterations and Antigenic Patterns of RS Infections

Immunohistoarcheology - Using the enzyme-linked immunohistologic technology developed in this laboratory, and pathologic specimens derived from culture proven cases of RS virus infection obtained from collaborators, it was possible to define a constellation of histologic changes accompanying fatal RS virus disease. Previously unrecognized endobronchial lesions were detected which include a lytic focal endobronchial mucosal dissolution which leaves small characteristic mucosal ulcerations. This focal lytic endobronchitis is unique in that there is a paucity of responding leukocytes despite a large amount of viral antigen and tissue destruction. These changes are likely the precursor lesions to the changes recognized by Zinserling which in his studies could not be definitely linked to RS virus.

Involvement of the peribronchial mixed mucous and serous glands by RS virus has not been described previously. Immunochemical staining revealed viral antigen in such tissues. Of interest, the lungs of infants or elderly individuals who died of RS virus disease revealed minimal structural alteration of the acinar tissues despite distinct viral inclusion bodies and the presence of as much viral antigen as is seen in tissue culture cells after 96 hours of experimental infection. The physiologic consequences of viral replication in the mucus glands are not known but may contribute to a decreased efficiency of the local pulmonary defense mechanisms and perhaps contribute to the bronchiolar plugging observed in RS virus disease. These observations suggest that RS virus may have an effect on the function of large areas of the lung not actually infected by the virus.

Although recognized as responsible for the development of endobronchial syncytial giant cells since the work of Adams, RS virus has not been considered a cause of giant cell pneumonia. Analysis of fatal RS virus disease indicates that in the immunocompromised patient there is an unusual presentation of a giant cell pneumonia with unusually large (up to 70 u) and occasionally multiple eosinophilic cytoplasmic inclusions. In this form of RS virus infection the amount and density of antigen present is comparable to that seen in the most extensive experimental infection of cell cultures.

Staining of infected cell cultures with certain antisera demonstrated a hyaline cytoplasmic ring rich in RS virus antigen at the periphery of syncytial giant cells. This rim was resistant to dissolution even after extensive necrotic alteration of the remainder of the giant cell. This property of RS virus-induced giant cells makes possible the detection of residual ring-like structures within the endobronchial plugs of patients with fatal RS virus disease. These structures are visible in routine sections of lung tissue and are sufficient to suggest a diagnosis of RS virus disease.

4) Influence of Oligopeptides and Ribovirion on RS Virus Replication

Background - Although prophylaxis through vaccine is deemed the most effective method of disease control, there are circumstances in which chemotherapy would be useful. We have examined the effects of selected oligopeptides *in vitro* and *in vivo* for their ability to suppress RS virus replication.

Amino Carbobenzoxy (cbz) oligopeptides and homologous non-cbz oligopeptides were assayed for their capacity to effect plaque reduction at various concentrations. The peptides cbz-phe-phe, cbz-phe-phe-arg, and cbz-phe-phe-gly caused 50% plaque reduction at concentrations of 0.3 to 0.1 u molar; however, these substances were relatively toxic to the cells at these concentrations and the suppression was probably related to this toxic effect.

Ribavirin a guanosine monophosphate competitor was assayed for its capacity to effect plaque reduction. This compound caused a 50% plaque reduction at a concentration of 8-14 ugm/ml in cell culture. *In vivo* experiments in cotton rats showed that 500 mg/kg/day lowered pulmonary and nasal viral replication approximately 90%.

5) Characterization of Polypeptides of Respiratory Syncytial Virus

Studies have recently been initiated to characterize the polypeptides present in RS infected cells by polyacrylamide gel electrophoresis. The purpose of these studies is three-fold: (i) to be able to compare polypeptides made in wild-type and vaccine candidate strains as a possible marker of the defectiveness of such mutants; (ii) to identify the surface glycoproteins of RS virus; (iii) to develop an immunoprecipitation assay to measure the response of animals and volunteers to the various viral antigens so that vaccine candidates can be compared in terms of the efficacy of induction of antibody to the relevant surface antigens. As an adjunct to this we are attempting to isolate the surface glycoproteins so that we can measure the immune response to these surface antigens directly.

PUBLICATIONS

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00179-02 LID

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

MOLECULAR BIOLOGY OF RESPORATORY AND GASTROINTESTINAL VIRAL PATHOGENS STUDIED BY DNA RECOMBINANT TECHNOLOGY

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH
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SECTION
RESPIRATORY VIRUSES SECTION

INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	1.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

DNA sequences corresponding to influenza viral gene segments that code for the non-structural proteins (NS, gene 8), matrix protein (M, gene 7), neuraminidase (NA, gene 6) and hemagglutinin (HA, gene 4) were cloned in a plasmid in *E. coli*. The cloned NS DNA was employed to map mRNA coding for the non-structural proteins derived from gene 8 using the S1 nuclease technique. Two separate mRNA's different in size were identified, one responsible for the NS1 and the other for the NS2 polypeptide. The NS1 mRNA appeared to be a continuous transcript, while, the NS2 mRNA contained an interruption produced by splicing. Cloned DNA derived from the genes for the NS proteins, NA and HA contained a complete representation of their respective genes. These cloned DNA genes are thus suitable for study of all phases of influenza gene regulation and expression. The cloned sequences also can be used to attempt to convert DNA into functional influenza virion RNA. Also, heterogeneous, non-viral oligonucleotides corresponding to the 5'-terminus of viral mRNA were present in cloned DNA suggesting that a variety of cellular RNA sequences are utilized to prime influenza viral mRNA synthesis.

Objectives

(1) To employ recombinant DNA technology to study the genes of influenza A virus and their functions. (2) To elucidate the pattern of antigenic variation among influenza virus strains by analysis of cloned DNA sequences that code for the viral surface antigens. (3) To study the mechanism of viral gene expression at the transcriptional level by nucleotide sequence determination of cDNA copies derived from viral mRNA. (4) To examine cloned DNA for viral protein synthesis in eukaryotic cells as well as in *E. coli* (harboring influenza virus genes). (5) To determine the antigenic determinants in relation to antigenic variation and to delineate the functional domains of the viral surface proteins. (6) To develop procedures for conversion of cloned viral DNA back to the virion RNA with the intent of constructing and isolating defined mutants for use in experimental studies and possibly, immunoprophylaxis.

Methods

(1) Preparation of influenza viral RNA segments - extraction of influenza virion RNA, fractionation of cytoplasmic mRNA from virus-infected cells.

(2) Synthesis of influenza viral cDNA strands - reverse-transcription of the virion RNA segments using a specific DNA primer which is complementary to the conserved 3'-end of virion RNA segments and reverse-transcription of the cytoplasmic viral mRNA segments using oligo (dT) as a primer.

(3) Cloning influenza viral DNA segments in *E. coli* plasmid pBR322.

(4) Characterization of cloned viral DNA molecules by restriction enzyme cleavage.

(5) Analysis of nucleotide sequence using the method of Maxam and Gilbert and the method of Maat and Smith.

(6) Immunoprecipitation and/or radioimmunoassay for viral peptides synthesized in *E. coli* containing influenza virus genes or in eucaryotic cells transfected with cloned viral DNA.

(7) Introduction of cloned viral DNA into mammalian cells by transfection using deletion SV40 DNA as a vector and by co-transformation using the thymidine kinase gene from herpes virus simplex.

(8) Conversion of cloned viral DNA back to influenza virion RNA - "marker rescue" of defined ts mutants of influenza virus by cloned DNA and sero-selection of a different influenza subtype using cloned DNA coding for the surface antigens.

Background

The genome of influenza virus consists of eight separate RNA segments that vary from 900 to 2400 nucleotides. The virion RNA segments are negative strands, that is, their complementary RNA transcripts become associated with the polyribosomes and are translated to produce viral specific proteins. With the exception of viral RNA segment 8 which encodes two different non-structural proteins, each RNA segment seems to code for a specific viral component and to be responsible for a specific viral function. The functional role which these viral proteins play in viral replication and maturation is only partially understood. Complementation-reassortment analysis of temperature-sensitive (ts) mutants of influenza virus demonstrates that at least eight complementation groups of ts mutants have been isolated and characterized.

One feature distinguishing influenza virus from other non-oncogenic RNA virus is that the functioning of the host nuclear RNA polymerase I is required for virus replication, expecially for viral RNA transcription. Studies using an in vitro assay system show that capped eukaryotic mRNA's donate their 5'-terminal methylated cap and a short stretch of internal nucleotide to the 5' end of the viral RNA transcripts. Although recent results suggest that a similar mechanism appears to operate in vivo, more direct evidence for the host RNA involvement in the initiation of influenza viral mRNA transcription is still lacking. In addition, the sequence specificity and the length of the transferred RNA segment for the initiation of mRNA transcription are not known. Many questions relating to the regulation of transcription such as the biosynthesis of the two different mRNA's derived from a simgle viral gene segment (gene 8, the smallest RNA segment) remain to be answered.

Another important feature of influenza virus is antigenic variation, or the emergence of viral subtypes (antigenic shift). Other phenomena involving antigenic variation of a lesser extent within a subtype (antigenic drift) are also observed. Such antigenic instability results in epidemiologically significant differences in surface antigens (hemagglutinin and neuraminidase) seen in the influenza A virus subtypes. When this type of change is abrupt and extensive (antigenic shift) a pandemic ensues. As a consequence, the continuing change of influenza strains has rendered ineffective efforts to vaccinate man against major new antigenic variants.

In view of the medical importance of influenza viruses there is a need to broaden our knowledge of the genetics of these viruses in order to design ways to prevent pandemics. One approach is to use recombinant DNA technology that has been well-developed and has proven valuable in elucidating gene organization and expression in other appropriate host systems. Our general plan for this approach includes two phases. The first phase involves cloning each of the eight influenza gene segments using the E. coli K12-plasmid system (approved MUA #91). The second phase of development involves the use of purified clones of influenza DNA sequences to examine viral gene expression in animal cell culture and to produce vRNA in eukaryotic cells. To carry out this second phase we propose to utilize defective SV40 (lacking the late region of the geonme that codes for capsid proteins) for construction of influenza-SV40 hybrid DNA molecules (approved MUA #110). Introduction of the appropriate influenza-SV40 hybrid DNA molecules into eukaryotic cells may lead to transcription or translation of influenza viral DNA depending upon its orientation of insertion. Our goal is to devise procedures which would permit conversion of influenza DNA back to an influenza RNA negative strand and eventually transfer such RNA into an influenza virus. In this manner, stable site-specific mutations, such as deletions, induced in the cloned DNA might be transferred back to the influenza virus. Thus, it may be possible to develop stable mutants for experimental study and for use in immunoprophylaxis.

Major findings

(A) Influenza Viral Gene Segments Cloned in E. coli Plasmid

As an initial step in this study we devised a procedure for obtaining double-stranded DNA sequences corresponding to each of the influenza viral RNA segments. Initially, positive and negative cDNA strands were separately prepared by reverse-transcription. The positive strands of a cDNA were transcribed from genomic RNA segments by using a specific primer of dodecamer DNA sequence which is complementary to the conserved 3'-terminal nucleotides of the virion RNA. The negative strands of cDNA were transcribed from cytoplasmic viral mRNA segments by using an oligo(dT) primer. The single stranded DNA molecules from both preparations were annealed to generated double-stranded DNA segments. The *in vitro* synthesized influenza DNA segments were inserved into plasmid pBR322 at the specific Pst I site using the dG-dC tailing technique. The hybrid DNA molecules were used to transform recipient *E. coli* and transformants containing influenza gene sequences were identified by hybridization. In this manner, we have obtained putative full-length influenza virus DNA segments corresponding to genes coding for the nonstructural proteins (gene 8), the matrix protein (gene 7), the hemagglutinin (gene 4) and the neuraminidase (gene 6). This approach should be generally applicable to other influenza virus genes as well as to other negative strand RNA viruses.

(B) Strand Orientation and Restriction Enzyme Maps of Cloned Influenza Viral DNA Segments

Initially, the influenza virus specific DNA sequences were characterized by restriction enzyme digestion. The digests of these DNA fragments resulting from treatment with restriction enzymes that produced a single cleavage or multiple cleavages were analyzed by polyacrylamide gel electrophoresis. The restriction enzyme cleavage sites were positioned on the linear DNA map of each DNA segment and oriented with respect to the 3'-terminus of the virion RNA. Generally, orientation was carried out by forming duplex DNA molecules between the 5'-labelled plus cDNA strand and an excess of the cloned DNA segment and cleaving the duplexes with a restriction enzyme. The labelled terminal fragment which positioned the 3'-end of virion RNA was identified by gel electrophoresis. From these experiments we established the restriction enzyme cleavage maps of cloned DNA sequences corresponding to 4 genes of influenza virus strain A/Udorn/72 (H3N2).

(C) Mapping mRNA's for Non-structural Proteins NS1 and NS2 on RNA Segment 8 of Influenza Virus Genome

RNA segment 8 of the influenza virus genome is unique in coding for two different polypeptides NS1 and NS2. These polypeptides are synthesized from separate mRNA species. By using the cloned DNA derived from virion RNA segment 8 (NS DNA) the template locations for the two mRNA transcripts have been mapped by the S1 nuclease method. These data indicate the body of the NS1 mRNA maps at 0.05-0.95 units of the cloned NS DNA and the body of the NS2 mRNA maps at 0.59-0.95 units, suggesting that the two mRNA's share the same poly (A) addition site at their 3' ends. The template locations of the mRNA's on the viral genome segment were confirmed by hybrid-arrested translation experiments using fragments of the cloned NS DNA to inhibit the synthesis *in vitro* of NS1 and NS2 polypeptides. In addition, in these translation experiments certain DNA fragments brought about premature termination of the NS1 polypeptide. From these data, it could be estimated that the termination of translation of NS1 is at 0.76 map unit. Thus, the coding regions of the two mRNA's overlap. Peptide mapping experiments indicated that NS1 and NS2 do not share methionine- or leucine-containing tryptic peptides. These results indicate that translation of the NS2 mRNA occurs in a reading frame different from that used for NS1.

(D) Sequence Determination of Interrupted and Uninterrupted mRNA's Coding for the Two Overlapping Non-structural Proteins of Influenza Virus

Experiments using primer extension were performed to determine more precisely the nucleotide sequences at the 5'-terminus of the NS2 mRNA. From nucleotide sequence determination it was found that the NS2 mRNA contains an interrupted sequence of 473 nucleotides. The nucleotide sequences at the junctions of the interrupted segment are similar to those of the consensus sequences at the splicing sites of intervening regions in eukaryotic mRNA's. In contrast, the NS1 mRNA appears to be continuous and therefore is a linear transcript of the gene segment. The first 56 virus-specific nucleotides at the 5'-end of the NS2 mRNA are the same as those found at the 5' end of the NS1 mRNA. This leader sequence of the NS2 mRNA contains the initiation codon for protein synthesis and codes for 9 animo acids which are common to NS1 and NS2 popypeptides. Both mRNA's contain heterogeneous non-viral oligonucleotides at their 5'-ends (see Major finding F).

(E) Determination of Completeness of Cloned Influenza Viral Genes

Complete influenza viral DNA sequences corresponding to each of the eight genes should be useful for further biologic experiments in elucidating regulation and expression of viral genes. Sequence analysis was carried out to determine the terminal nucleotides of the cloned viral DNA segments. Results from these studies showed that sequences at both termini of vRNA were fully represented in clone pFV88 (gene 4), pFV101 (gene 6) and pFV26 (gene 8) indicating that the cloned DNA contains the complete information present in the viral RNA segment coding for the hemagglutinin, the neuraminidase and the non-structural proteins, respectively. These experiments further demonstrate that our procedures for cloning influenza virus gene segments can be employed to produce complete copies of viral DNA which should have all the control signals for viral gene expression in eukaryotic cells.

(F) Elucidation of Non-viral Oligonucleotides at the 5'-terminus of Cytoplasmic Influenza Viral mRNA

As just described, we cloned influenza viral DNA duplexes formed between cDNA strands separately synthesized from virion RNA and from cytoplasmic viral mRNA produced in infected cells. Size analysis of cDNA strands synthesized by reverse-transcription and sequence determination of cloned DNA showed that the cloning procedure yielded complete copies of influenza genes and their corresponding mRNA. This procedure, therefore, provided material suitable for analysis of nucleotide sequences present at the 5'-terminus of individual cytoplasmic mRNA species. Sequence determination of terminal nucleotides from five independently isolated viral DNA segments showed that additional oligonucleotides were covalently linked to the 5'-terminus of viral mRNA transcripts. The sequences of these additional nucleotides varied among DNA clones of the same gene and of different genes as well. These inserts also varied in length. The heterogeneity of these sequences suggests that they were derived originally from cellular RNA molecules. These findings provide evidence that cellular RNA sequences are utilized to prime influenza viral mRNA transcription in infected cells.

Publications

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LABORATORY OF MICROBIAL IMMUNITY

1980 Annual Report
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PHS-NIH
SUMMARY STATEMENT

ANNUAL REPORT OF THE LABORATORY OF MICROBIAL IMMUNITY NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

October 1, 1979 to September 30, 1980

Richard Asofsky, M.D.
Chief, Laboratory of Microbial Immunity

Research in this Laboratory is mainly concerned with the differentiation of lymphoid cells and with basic mechanisms controlling cellular and humoral immune responses to microbial and tissue antigens. Selected viruses, blood parasites, bacterial polysaccharides and lipopolysaccharides, as well as "integrated" leukemia viruses and histocompatibility antigens are used. Several genetic studies are in progress and include the identification and distribution in inbred mice of genes controlling synthesis and expression of several polypeptides of leukemia viruses, those controlling differentiation of lymphocytes, especially of T-lymphocytes, and those controlling antibody formation to pneumococcal and other polysaccharides. Assembly of immunoglobulins is studied in neoplastic B-cells, adopted to and cloned in vitro. Somatic hybridization of immune cells to drug-marked myeloma lines is used to obtain monoclonal antibodies to parasites and to differentiation antigens. Several strains of "autoimmune" mice are under active study, as is induced experimental encephalomyelitis in guinea pigs.

OSCILLATORY PATTERNS GENERATED DURING THE ANTIBODY RESPONSE

Oscillations in numbers of antibody-producing plaque-forming cells (PFC) have been observed after immunization with bacterial lipopolysaccharide (LPS) and levan antigens. Although theoretical studies indicate that such oscillatory patterns may be due to the expression of negative feedback, the mechanisms involved are not understood completely. The adjuvanticity and toxicity associated with the lipid A component of LPS does not appear to play a dominant role since oscillation was observed in both C3H/HeN and C3H/HeJ strains of mice; the latter lacks cell receptors for lipid A and hence is refractory to its pharmacologic effects. The results of preliminary studies conducted with athymic nude mice suggest that oscillatory patterns are dependent on the presence of thymus-derived (T) lymphocytes. Furthermore, the formation of auto-antiidiotypic antibody also appears to be involved. Studies are underway to determine whether T cells exert a suppressive effect on this process by providing "help" for the production of auto-antiidiotypic antibodies.
(Drs. J. Hiernaux, P. J. Baker, K. B. Schroer, B. Prescott, C. Bona, C. DeLisi, and J. A. Rudbach).

A MATHEMATICAL MODEL OF B CELL DIFFERENTIATION

A model has been proposed which uses a modified form of the X→Y→Z differentiation scheme of Sercarz and Coons, time delays in differentiation, and antibody feedback. The differential equations in this determanistic model predict a

"staircase" growth in antibody forming cells (AFC) after a first injection of antigen, and rapid oscillations in AFC's thereafter. Although differentiation in T-cell dependent responses was not addressed, many of the results found by Hiernaux, et. al. (above) were predicted.
(Drs. Grossman (Weizmann Institute, rehovat, Israel) DeLisi (LTB, NCI) and Asofsky)

EFFECTS OF MITOGENS ON VARIOUS B CELL CHARACTERISTICS USING BALB/c LYMPHOMA LINES AS MODELS

Several spontaneously derived BALB/c B lymphoma lines previously have been reported to express various B cell differentiation markers. K46, L10A, BALENTL 17, and X16C were S-IgM$^+$, Fc receptor$^+$, Ia$^+$ and C3$^-$. A20 expressed S-Ig$^+$ (light chain only ?), Fc receptor$^+$, Ia$^+$ and C3$^-$. We examined several B cell polyclonal activators including lipopolysaccharide (LPS) and dextran sulfate (DS), for their ability to stimulate these tumor cell lines. 96 hrs after LPS stimulation X16C but no other cell lines showed a significant increase in the expression of S-IgM and a minimum change in S-IgD expression while there was no significant effect on the ^{3}H-thymidine incorporation. However, LPS has no effect on the secretion of surface IgM of X16C; as determined by radioimmunoassay or a reverse plaque forming cell assay. In an attempt to induce X16C to switch the expression of surface IgM to other classes of Ig heavy chain, nonspecific T cell factors such as TRF (thymocyte replacing factor) or AEF (allogeneic effector factor) were added into cultures with or without LPS. These T cell factors alone enhanced the expression of S-IgM but no other classes of Ig heavy chain. One of the striking effects of the factors was the 2-3 fold increase of Ia antigen expression detected by ATH anti ATL antiserum. (Drs. Kim, Kanellopoulos-Langevin, and Chused).

GENETIC DISSECTION OF AUTOIMMUNE FACTORS IN NZB DISEASE

NZB mice have multiple abnormalities of both T and B lymphocytes. B cells have a reduced cell surface ratio of IgD/IgM and their progeny secrete high levels of IgM. T cells from NZB are abnormal in generating cytotoxic T lymphocytes to H-2-compatible cells following primary stimulation *in vitro*. Mice from genetic crosses between NZB and B10.D2 were examined to determine the same or different genes governed the T and B lymphocyte abnormalities. Spleen cells from these mice were examined for cell surface expression of IgD and IgM, rates of IgM secretion, and their capacity to generate CTL to H-2-compatible cells. The results of this study demonstrated that each of these abnormalities is determined by different sets of genes. The altered ratio of cell surface IgD/IgM is a recessive trait whereas alterations in plasma cell frequency, rates of IgM secretion, and T cell responses are under multigenic control. Immunologic abnormalities of NZB mice thus reflect multiple derangements in genes which control the functions of T and B cells. (Drs. Davidson, Morse, and Chused).

GENETIC STUDIES OF LEUKEMIA VIRUS EXPRESSION

In collaborative studies with Drs. Janet Hartley and Christine Kozak of LVD we have shown that F/St mice produce high levels of both ecotropic and xenotropic MuLV. The high ecotropic virus phenotype of F/St, like that of AKR, C58 or C3H/Fg, behaves as a dominant trait in genetic crosses with other strains of mice and is governed by multiple virus loci. By contrast, the high xenotropic virus phenotype of this strain behaves as a recessive trait in all crosses examined to date. Xenotropic virus is inherited in these crosses as it can be induced from cells which spontaneously produce no virus and we have mapped the inducibility locus to chromosome 1. Studies of F_1 mice backcrosses to F/St showed that suppression of xenotropic MuLV is governed by a single locus which is not on a known chromosome and which we are attempting to map. Parallel findings have been made with other strains which produce moderate levels of xenotropic MuLV. These results suggest that expression of xenotropic MuLV is normally restricted by a transactive gene, the product of which is abnormal or not present in F/St mice. (Drs. Morse, Hartley (LVD) and Kozak (LVD).

CATARACT FORMATION IN ACUTE EXPERIMENTAL ENCEPHALOMYELITIS

Young guinea pigs in the early acute stages of experimental encephalomyelitis develop cataracts. These do not resemble rare spontaneously arising cataracts of guinea pigs pathologically. the cataracts were associated with acute forms of disease, and were often seen to resolve as disease progressed to the chronic form. (Dr. Stone).

ONTOGENY OF DIFFERENTIATION IN THE THYMUS

In most strains of mice, about 10% of adult thymocytes are Lyt 1^+2^-, and 90% Lyt 1^+2^+. A study of the ontogeny of this distribution was undertaken. In 16 day embryos, about 50% of thymocytes are Lyt 1^+, while at most 15% are Lyt 2^+; e.g. Lyt 1^+2^- cells appear in the thymus before Lyt 1^+2^+ cells. In 17 day embryos, more than 90% of cells are Lyt 1^+, and more than 70% Lyt 2^+ or Lyt 3^+. It thus appears either that Lyt 1^+23^+ cells are the progenitors of Lyt $1^+2,3^+$ cells or that Lyt $1^+2,3^-$ cells and Lyt $1^+2,3^+$ cells are separate cell lines. The accepted scheme of differentian, Lyt $1^+2,3^+$ Lyt $1^+2,3^-$ and Lyt 1^- $2,3^+$ is therefore incorrect. (Drs. Mathieson, Rosenberg, Ms. Sharrow, (NCI), Dr. Hammerling (Sloan-Kettering Institute)).

SYNERGISTIC ACTIVITY OF THYMOCYTE SUBSETS

Flow microfluorometric (FMF) analysis of lymphocyte cell surface antigens, and functional characterization of thymocyte subpopulations obtained after peanut lectin (PNA) agglutination has been extended. Characterization of the functional capacity of thymocyte subpopulations obtained by PNA agglutination and positive selection for the Lyt 2+ phenotype has indicated that PNA nonagglutinated thymocytes could be subdivided both functionally and phenotypically into Lyt 1^+23^- and Lyt 123^+ subpopulations. The ability of these subsets to synergize in mixed lymphocyte reactions (MLR) and graft-versus-host (GVH) reactions has been demonstrated. In addition, the capacity for proliferative responses to mitogens, antigens and lymphokine stimulation has been characterized for these subsets.

ADMINISTRATIVE

Dr. Sanford H. Stone joined the Laboratory this year, and has continued research on immune encephalomyelitis in guinea pigs. Also joining the Laboratory were Dr. Teruaki Hamano, a Visiting Fellow from Japan, Dr. Gerard Chaouat, a Guest Scientist from France, Dr. Vijaya Manohar, a Visiting Fellow from India, and Dr. Jacques Hiernaux, a Visiting Fellow from Belgium.

Drs. Phillip Munoz and Kenneth Schroer completed research associateships. Dr. Schroer is an expert in the Pathology Branch, NCI. Dr. Munoz will complete his residency in Pathology at the University of Kansas.

Dr. Christopher Bever, a neurologist, joined the Laboratory in July as a Research Associate. Dr. Paul Beining spent a sabbatical year in Dr. Phillip Baker's laboratory, working on antibody responses to lipoteichoic acid antigens.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00134-18 LMI

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Control of Immunoglobulin Synthesis in Mice

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. Asofsky	Laboratory Chief	LMI/NIAID
OTHER:	K. J. Kim	Visiting Associate	LMI/NIAID
	H. C. Morse III	Senior Investigator	LMI/NIAID
	B. J. Fowlkes	Microbiologist	LMI/NIAID
	P. E. McKeever	Senior Investigator	SN/NINCDS
	C. Kanellopoulos-Langevin	Visiting Associate	LMI/NIAID
	Y. Rosenberg	Visiting Associate	LMI/NIAID
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	R. Merwin	Senior Investigator	LCMB/NCI
	M. Neiders*		
	D. H. Sachs	Senior Investigator	IB/NCI
	S. O. Sharrow	Chemist	IB/NCI
	A. Rafai	Staff Fellow	NIAMDD

COOPERATING UNITS (if any)

*SUNY At Buffalo, Buffalo, New York

LAB/BRANCH

Laboratory of Microbial Immunity

SECTION

Experimental Pathology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	2.0	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

SAMM 368, a myeloma which secretes IgG2b and IgA paraproteins, assembles heavy and light polypeptide chains of both immunoglobulin (Ig) classes correctly without molecular mixing. The IgG2b paraprotein is secreted five times as rapidly as IgA. The latter is assembled in the cytoplasm at a high rate but is released (secreted) slowly.

Immunologic and immunogenetic conditions which are required for the differentiation of plasma cells making each of the classes of immunoglobulin have been studied. Conditions can be found which favor either IgM or IgG synthesis.

Project Description:

This project is designed to elucidate the genetic and developmental factors which regulate the development of B lymphocytes and plasma cells which are their progeny. Certain very interesting neoplastic cell lines have been established in our Laboratory, so work has focussed on studies of lymphoid tumors as well as on normal differentiation.

1. SAMM 368, A Double Secretor.

(Drs. McKeever, Neiders, Kim, Morse, and Asofsky)

In a large study of plasma cell tumors in BALB/c mice one tumor was found which produced two classes of immunoglobulin (IgG2b and IgA) and two idiotypes. Chemical analysis of light chains from the two paraproteins showed a 30% difference among the 35 amino terminal residues. This tumor was established *in vitro*, and a study of intracellular synthesis, assembly, and secretion of its immunoglobulin products is ongoing. Using methods described by Scharff and his colleagues it has been found that most clones incorporate radiolabeled amino acids into IgA and IgG2b, but one line has been found which synthesizes only IgA, another which synthesizes only IgG2b. In all cases the immunoglobulin is correctly assembled, both within the cell and in extracellular secretions. It has proved impossible so far to chemically separate the two light chains, although the light chain associated with the α chains is, as expected, a dimer, which is not covalently linked to heavy chain and the light chain associated with the γ chain is a monomer covalently linked, also as expected. Mixed molecules which are seen in hybrids of two myelomas are not seen. The molecular mechanism which accounts for this correct arrangement of molecules is not clear. Pulse-chase experiments show that the IgG2b is secreted five times more rapidly than the IgA. The cells make IgA which is stored in the cytoplasm before secretion. Work in the near future will examine whether this arrangement is simply fortuitous in this cell line, or whether there are other molecules in the cell sap of this tumor which block or impede incorrect arrangement.

2. Interaction Between Alloantigens and Fc (IgG) Receptors on the Surface of BALB/c B Cell Lymphoma Lines.

(Drs. Kanellopoulos-Langevin, Sachs, Rafai, and Asofsky; Ms. Sharrow)

Using several cell lines from a panel of BALB/c B cell lymphomas, we have addressed the question of a possible relationship between Fc receptors and alloantigens on the surface of B lymphocytes. This problem arose from a controversy in the literature among studies on normal spleen cells. Some authors found an inhibition of Fc receptors only when using antisera directed against Ia or Ia-like antigens. They thus postulated a "specific" ligand-induced interaction between Fc receptors and Ia antigens. Conversely, other laboratories observed blocking of Fc receptors after interaction of antibodies (or $F(ab)'_2$ fragments thereof) with any antigen on the surface of B cells.

We have used the B cell tumors as models because they have several advantages over heterogeneous spleen cell populations: (1) They are grown continuously *in vitro* and are thus free of antibodies or immune complexes bound to their surfaces. (2) They have been cloned and are highly homogeneous. (3) We have several tumor lines with different surface phenotypes which might be equivalent of distinct normal B cell subpopulations. A normal BALB/c spleen cell sample was studied in parallel in every experiment.

Using an EA rosetting assay to detect Fc receptors, we first observed that rosettes on spleen cells were inhibited by anti-Ia and anti-LyM as well as anti-H-2D sera. Two tumor lines (K46 and X16C) gave a similar pattern, whereas, two other cell lines (A20, M12) were inhibited significantly only by anti-Ia or anti-LyM sera. Quantitative absorptions showed no major difference in the amount of H-2 antigens per cell on A20 and K46. In order to obtain a fuller picture, we subsequently studied inhibition of Fc receptors detected by two other assays e.g. binding of fluoresceinated heat-aggregated human γ globulins and binding of fluoresceinated chemically cross-linked mouse IgG). The amount of fluorescence per cell was measured using a fluorescence activated cell sorter (FACS II). EA rosetting and both fluorescence assays were done on the same day on the same cells. Anti-Ia and anti-H-2D sera were equally inhibitory of both fluorescence assays on normal spleen cells and all tumors. Pre-incubation of the cells with LPS did not yield any significant inhibition of any of the three assays.

Experiments are now in progress to study the effect of the binding of non-antibody molecules (such as lectins) to the B cell membranes. We are also planning to study inhibition of Fc receptors (tested by three different assays) by anti-Ia and anti-H-2 IgM antibodies (from hybridomas) in order to completely rule out interactions of Fc portions of alloantibodies with Fc receptors.

3. Regulation of Immunoglobulin Heavy Chain Gene Expression.

(Dr. Rosenberg, Ms. Lieberman, and Dr. Asofsky)

(a) B Cell Activation by "Class-Specific" Helper Cells:

In order to gain some understanding of the helper mechanisms operating following immunization with T-dependent antigens, purified antigen-primed T cells were cultured with normal unprimed B cells both *in vitro* and following adoptive transfer and the wells and mice were analysed for total class-restricted IgFC. The *in vitro* results indicate that (a) T cells derived from antigen primed mice have the ability to trigger unprimed B cells into IgFC of the particular isotype corresponding to that which the antigen would have produced *in vivo*; (b) two helper cells plus the priming antigen are required for observed restricted responses; (c) T cells from spleens of mice suppressed for IgM production lack such helper cell activity, and (d) responses are abrogated by treatment of T cells with anti-LyT 1 antisera. In addition, responses obtained at limiting dilutions of T cells are compatible with the requirement for both helper cells for B cell induction.

(b) Genetic Control of Class-Restricted B Cell Activation:

(1) Preliminary experiments employing allotype congenic mice, e.g., BALB/c CB20, and BAB14 suggest that such Ig-specific T cells are specific for V_H determinants.

(2) Using antigens, the responses to which are under Ir gene control, e.g., GAT, preliminary experiments indicate that IgFC are produced only when the B cell plus macrophage populations and the T cell populations are both derived from responder strains.

(c) B Cell Induction Following Non-Specific T Cell Activation:

It is clear that any perturbation of the immune system, whether a result of mitogen injection, antigen administration, or injection with pathogenic organisms, has marked effects on the levels of splenic IgFC, which is found to be dependent on prior T cell triggering. In order to evaluate possible mechanisms of B cell activation by T cells or their T cell derived factors, mice were exposed to a variety of specific or non-specific T cell activators and their spleens assayed for total class-specific IgFC. The results are summarized as follows: Response patterns may be random, restricted, rapid, or prolonged, and reflect the existence of several different mechanisms of T cell help for Ig production, supporting the increasing evidence for functional heterogeneity and synergy in the helper T cell pool.

T cell Activation	Nature of Response	IgM	IgG1	IgG2	IgG3	IgA
Con A	Rapid, D3 Peak	+++	+++	+++	ND	+++
17XL malaria	Delayed, D7	++++	++++	++++	ND	++++
ALS	D6	+	++++	++	ND	-
GVH	D6	+	++++	++	+	-
CFA	Delayed/ Prolonged	±	++	++	ND	±
Colchicine + soluble HGG	D5-6	+	++++	++	—	—
PHA	—	—	—	—	—	—

In summary, the existence of helper cells which are triggered by isotypic determinants on B cells of a particular specificity and, in turn, have the ability to stimulate B cells having a different specificity but bearing a similar isotype, a situation which occurs in the present *in vitro* cultures, is strikingly similar to the idiotype network and suggests that regulation of immune responses may result also in part from an "isotype network".

4. Studies of Murine Lymphoid Tumors as Models of Normal Lymphocytes.

(Drs. Kim and Kanellopoulos-Langevin)

It has been well established that chronic lymphocytic leukemia (CLL) in humans is a clonal proliferation of B cells. Only in recent years have there been a few reports of murine B lymphoid cell lines with defined differentiation markers.

More than thirty BALB/c lymphoid tumors, most of which arose spontaneously, were screened for the expression of surface Ig (IgM, IgD, IgG_1, IgG_2, IgA, κ, and λ). Six of these tumors were adapted into culture successfully and were characterized for the expression of Ig, Fc (IgG) receptor, complement receptor, and Ia antigens. Four lines (K46, X16C, L10A, BALENLM 17) express IgM, Fc, Ia, and C. On the other hand, A20 expresses Ig (of undetermined isotype), Fc, Ia, and C, while M12 are $Ig^{\pm}$,Fc^{+} and Ia^{-}. The membrane IgM was shown to be synthesized by these tumors using radio-labeled amino acid precursors. We also showed the expression of IgD on K46, X16C, L10A using a ^{125}I-surface labeling technique. K46 tumors bearing Ia antigens were found to be a good source for preparation of Ia antigen for xeno-immunization in order to obtain an anti-Ia antibody recognizing road specificity.

Further, the controversial issue whether there is any clear relationship between Ia antigen and Fc receptor was examined using these Fc^{+}, Ia^{+} (K46 and A20) tumors and Fc^{+} Ia^{-} (M12) tumor cell lines as models. It appeared that there was no clear, unique relationship between Ia and Fc receptors.

We are currently analyzing the frequency of each heavy and light chain immunoglobulin on the spontaneous tumors using a Fluorescence Activated Cell Sorter (FACS), and are attempting to establish B lymphoid cell lines expressing heavy chain isotypes other than IgM or IgD.

T lymphoid tumors have been derived by 1-ethyl-1-nitrosourea or by estrogen. These tumors have been adapted in vitro and were confirmed to express various combinations of Lyt 1,2, TL antigen. Further, these tumors were shown to have a high glucocorticoid receptor regardless of the phenotypes of these tumor cell lines. These tumors were further used for the biochemical studies of Lyt antigens.

5. Theoretical Analysis of B Cell Differentiation.

(Drs. Z. Grossman, Weizmann Institute, Rehovot, Israel; C. DeLisi, LTB/NCI, and Asofsky)

A theoretical (mathematical) model has been proposed which uses the X→Y→Z model of Sercarz and Coons antibody feedback and time delays in differentiation to explain the "staircase" growth of antibody forming cells and oscillations in the number of antibody forming cells. So far this model has been applied to B cell antigens only, such as bacterial LPS. T-cell dependent antibody responses were not treated.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00135-06 LMI

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Properties of Immunoglobulin Secreting Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. C. Morse III	Senior Investigator	LMI/NIAID
OTHER:	R. Asofsky	Laboratory Chief	LMI/NIAID
	M. Potter	Senior Investigator	LCBGY/NCI
	J. Hartley	Senior Investigator	LVD/NIAID
	T. Mossman*		
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COOPERATING UNITS (if any)

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Plasmacytomas of NZB, BALB/c, and (BALB/c X NZB)F_1 mice were compared to determine what gentically determined factors, including murine leukemia viruses, contribute to the unique susceptibility of these strains to plasmacytoma induction. Polyclonal B cell activation may be the major determining factor.

A unique plasmacytoma of mice, closely resembling human Mott cell tumors, was detected during a study of BALB/c plasmacytomas. These cells produce, but do not secrete, IgA immunoglobulin.

Project Description:

NZB, BALB/c and (BALB/c X NZB)F_1 mice are the only strains in which plasmacytomas can be readily induced. Comparisons of plasmacytomas from these three strains and other strains indicates that susceptibility to plasmacytoma induction is a single recessive trait with NZB and BALB/c mice having the same or similar alleles at an unmapped locus determining susceptibility. Other loci appear to affect the rates of plasmacytoma induction and the frequency with which different immunoglobulin classes are produced by plasmacytomas. No definitive role for murine leukemia viruses could be established although a recombinant (MCF) virus was recovered from one tumor of a BALB/c mouse. This virus did not accelerate plasmacytoma induction in this strain.

A single mouse injected with cells infected in vitro with this virus developed a unique tumor resembling human Mott cell tumors. Cells from this tumor have markedly dilated endoplasmic reticulum filled with IgA immunoglobulin which is not secreted.

A series of NZB plasmacytomas were shown to secrete heavy chains with characteristics of both IgA and IgG3 immunoglobulins. The proteins bind to protein A, like normal IgG3 proteins, but have polypeptide mgss closely resembling normal IgA heavy chains.

Publications:

Morse, H. C. III, Hartley, J. W., and Potter, M.: Genetic considerations in plasmacytomas of BALB/c NZB, and (BALB/c X NZB)F_1 mice. In Potter, M., (Ed.), Progress in Mycloma, Elsevier-North Holland, New York, 263-281, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00136-08 LMI

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Functional Activities of Subpopulations of Thymus-Derived Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. J. Mathieson	Staff Fellow	LMI/NIAID
	R. Asofsky	Laboratory Chief	LMI/NIAID
OTHER:	S. O. Sharrow	Chemist	IB/NCI
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SECTION

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.1	1.6	0.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A new scheme of differentiation within the thymus is described. Lyt 1^+23^- cells comprise about 10% of normal adult thymus. Such cells are the earliest to appear in ontogeny. Lyt 1^+23^+ cells may be descendents, rather than progenitors of Lyt 1^+23^- cells. A new alloantigen, Ly 9, with two alleles, Ly 9.1 and Ly 9.2, is described. Ly 9 is found on all lymphocytes. Certain autoantibodies are described. One, Au-1 detects a subset of bone marrow cells, all thymocytes, and is absorbable by brain tissue.

Lymph node cells have been shown to interact synergistically with the 10% of thymocytes not agglutinated with peanut lectin in mixed lymphocyte reactions.

AKTB-1, a lymphoma of AKR mice, has been separated by fluorescent activated cell sorting into a B cell line (IG$^+$) which grows in follicles of the spleen, and a T cell line (Ig$^-$) which grows in lymph node and in interfollicular areas of spleen.

Project Description:

1. Lyt Antigen Expression on Normal Thymocytes and Selected Subpopulations.

Our analysis of thymic lymphomas indicated that these tumors had differentiated patterns of cell surface expression of Lyt antigens. Such differentiated patterns of Lyt antigen expression had been attributed by others to functional subsets of peripheral T lymphocytes. Furthermore, it had been assumed from antibody-dependent, complement-mediated cytotoxicity that all thymocytes were undifferentiated Lyt 123^+ cells. In contrast, we proposed that thymocytes differentiated while still in the thymus into functionally and phenotypically different subsets of cells and that these "committed" cells were in turn exported from the thymus. To examine these questions we developed flow microfluorometry (FMF) analysis for immunofluorescence (IF) of normal thymocytes and T cells and were able to detect a subsets of Lyt 1^+23^- cells which account for 10% of normal thymocytes which exist throughout the normal life span of mice. This work also demonstrated that the Lyt 1^+23^- subset of cells was increased to 50% in cortisone resistant thymocytes (CRT). Further the Lyt 1 expression was in fact shown to be increased on the larger cells in the CRT.

We then analyzed normal thymocytes by three techniques to attempt to enrich different subpopulations of thymocytes. These studies indicated that thymocytes separated by velocity sedimentation can be enriched for cells with increased Lyt 1 expression by selection of the largest cells. However, this method does not either enrich or deplete the Lyt 1^+23^- subpopulation. In contrast when thymocytes are separated by density gradient fractionation the subpopulations of Lyt 1^+23^- cells can be enriched in the low density fractions which compares to CRT. This enriched population is also like CRT because Lyt 1 bright cells are increased by this method.

The third method for fractionating thymocytes, peanut lectin (PNA) agglutination has proven the most useful because of its relative ease and reproducibility. Using this method, we have shown that agglutinated, PNA$^+$, thymocytes are comparable to the majority of thymocytes because they are high Thy 1^+, Lyt 123^+ and depleted of low Thy 1^+, Lyt 1^+23^- cells. Non-agglutinated PNA$^-$ thymocytes, approximately 7-10% of normal thymocytes, are highly enriched (30-60%) for Lyt 1^+23^- thymocytes and thus are comparable in phenotype to CRT. Furthermore, we have determined that there is a population of 6% of the Lyt 2^+, PNA$^-$ thymocytes which have acquired another marker, CTL which had been demonstrated previously to be expressed on cytotoxic effector (T_c) and cytotoxic precursor T cells (CTLp). Using the pre-enrichment with PNA and selective elimination with anti-CTL with parallel functional assays for CTLp, we were able to determine that a small Lyt 2^+ subset of thymocytes is differentiated for cytotoxic precursor function. This apparent differentiation is not complete in the thymus since all splenic CTLp can be eliminated with either anti-Lyt 2 or anti-CTL but only part of the CTLp can be eliminated in the PNA$^-$ fraction with anti-CTLp while all functional cells of this lineage, can be eliminated with anti-Lyt 2.

Further functional analysis of the PNA$^-$ fraction of thymocytes has indicated that these cells respond to stimulation with LAF. In particular the Lyt 1$^+$2$^-$ cells are the cells which respond to the proliferative stimulus with LAF.

Thus we have enriched and used subpopulations of normal thymocytes to demonstrate functional lineages of cells. We are currently working on the hypothesis that there are two distinct lineages of thymocytes Lyt 1$^+$23$^-$ cells and Lyt 123$^+$ cells. These differentiated lineages arise early in ontogeny, by day 16 gestation in the mouse (Mathieson, et. al., manuscript in preparation, and may be separated by functional and differentiative cell surface characterization.

2. Genetic Analysis of Lymphocyte Differentiation Markers.

(a) Genetic Analysis of Recombinant Inbred Strains of Mice for Lymphocyte Differentiation Markers.

In association with Dr. Roy Riblet et. al., we initially typed 14 NZB X C58 (NX8) recombinant inbred (RI) strains of mice at F 16-21 for Lyt 2.1/Lyt 2.2 expression and two closely associated Ig kappa light chain markers. This study confirmed that Lyt 2 is tightly linked to a gene controlling V_κ structure, since no crossovers were detected between Lyt-2, IgK-Pc or Igk-Ef1. These latter two markers are for particular light chain banding patterns observed in isoelectric fucusing gels from either phosphorlcholine-immunized or normal unimmunized mice respectively. However, in our initial examination of the NX8 strains we detected mice which were still heterozygous for both Lyt 2 and the light chain markers at the 16-18th generation.

We have now re-examined thirteen of the NX8 strains for both Lyt 2 and Lyt 3 expression as well as for Ly 9 allelic expression. These markers were examined because there is, to date, no evidence that Lyt 2 and Lyt 3 can be dissociated genetically. However, molecular biochemical evidence and differential sensitivity to enzymatic digestion on viable thymocyte surfaces, have indicated that the antigens may be expressed, at least in part, on separate molecules. We have observed no segregants in these animals which are now at generation 23-31.

We also examined these mice for Ly 9, because in analyzing the immune response to Lyt 2.1 and Lyt 3 in conventional immunizations for these antigens, we observed that the animals which responded to these antigens, routinely also responded to Ly 9. We therefore attempted to determine the interrelationship of these three antigens. In the NX8 strains there is no apparent linkage for Lyt 2,3 and Ly 9. Further studies to analyze linkage of Ly 9 to other markers in these strains are underway.

(b) Analysis of Lyt 2,3 and IgK-Ef Markers in Exceptional Mice.

Because of the close linkage for the Lyt 2 IgK-Ef markers seen in previous studies, the question of Lyt 2 control of light chain had arisen. We have analyzed an exceptional mouse which was derived from a mouse with a genetic crossover. Its progeny have indicated a gene order of Lyt 2-Lyt 3-IgK-Ef. While a crossover was observed in this mouse within the IgK-Ef marker region Lyt 2 and Lyt 3 still have not been observed to split genetically.

(c) Analysis of the Genetic Linkage of Ly 9.

To determine whether this antigen was genetically lined to other known antigenic determinants, we have analyzed two different sets of backcross mice (BALB/c X B6)F_1 X BALB/c and (NZB X B10.D2)F_1 X B10.D2. In particular, these studies have analyzed the mice for expression of H-2/TL, Ig allotype, Ly 9/Lgp 100, enzyme markers Dip-1 on chromosome 1 and Gpd-1 on chromosome 4, Mup and brown on chromosome 4. As indicated in the previous section on the NX8 RI lines, we have not seen linkage of Ly 9 with Lyt 2,3 or Igk markers. While not complete the backcross analyses indicate that Ly 9 may be linked either to Dip-1 (in the (NZB X B10.D2)F_1 backcross) or to Gpd-1 (in the (BALB/c X B6)F_1 backcross). There is no evidence in either of these crosses for linkage to any other marker except Lgp 100. With this marker, there is, so far, absolute colinearity; this is probably because these two markers may be identical, as discussed in the section on Ly 9 antigen characterization.

3. Characterization of New Lymphocyte Differentiation Markers.

In the course of immunizations for Lyt 1,2, and 3 differentiation markers, we identified two classes of additional lymphocyte markers which we have further characterized. These two classes of markers can be defined as thymocyte or lymphocyte cell surface markers which (a) have allelic forms of expression and defined strain distribution patterns, or (b) have no apparent allelic forms of expression and are expressed on the immunized animals own cells and thus are autoanbitodies.

(a) Analysis of Ly 9 Expression on Lymphoid Cells.

The first class of these serologically detected markers includes the lymphocyte differentiation marker Ly 9, which we have characterized extensively. This antigen has two allelic forms of expression which are seen as the common form, Ly 9.1, on lymphocytes of nearly all strains of mice except from strains related to C57BL/6, C57BL/10, C58 or two other strains derived from mice from the Lathrop-Loeb colony. These latter mice express the Ly 9b allele (as Ly 9.2) on their lymphocyte surfaces. This antigen is of particular interest because of its limited expression on lymphocyte precursors in the bone marrow. We are analyzing this point further by both elimination experiments and positive selection. The expression of Ly 9.1 correlates with a marker similarly characterized on lymphocyte cell surfaces by J. Ledbetter called Lgp 100 which is recognized by a heterologous monoclonal antibody. Because of the close similarities of this marker we are currently analyzing backcross mice for co-linear expression of Ly 9.1 and Lgp 100. This antigen also may have been recognized as T 100 by Gottlieb et al. We intend to further characterize the relationship between these molecules by blocking and pre-clearing with immunoprecipitation analysis. Because of minor differences between rats and mice with the anti-Lgp 100 and anti-Ly 9 reagents, we intend to examine further the potential toxonomic usefulness of this marker.

Because this antigen apparently is found only on thymocytes with the genetic material for its expression, we successfully have used this antigen, as well as Lyt antigens, for monitoring host-donor interactions of thymocytes in bone-marrow reconstituted, irradiation chimeras (S. O. Sharrow, A. Singer, B. J. Mathieson, and U. Hammerling, unpublished data).

(b) Characterization of Autoantibodies.

A second category of lymphocyte markers was detected in anti-Lyt immunizations which are characterized as autoantibodies (AU) or antibodies which react against host thymocytes. Cytotoxic, IgM, AU have been detected by others in immunizations of this type. However, there is marked heterogeneity in these AU when analyzed by flow microfluorometry (FMF) such that these AU display different immunofluorescent (IF) profiles. Upon further examination, the different profiles are reflected in the fine specificity of the AU. One of the AU, previsionally designated AU-1 detects a subset of bone marrow cells can be absorbed by brain tissue and is present on the majority, but not all, thymocytes. A second category AU-2 contains several AU with similar or related IF profiles. These profiles resemble the IF profiles seen with anti-Lyt 1, anti-TL (unpublished observations), anti-XenCSA or natural thymotoxic antibody from autoimmune mice. These AU on further examination have different fine specificities for different murine leukemia virus(MuLV)-associated antigens which can be detected on different heterologous MuLV infected cell lines. None of these AU have any apparent strain specificity, although this is being examined further.

(c) Characterization of Lectin Markers on Thymocytes.

Because of the usefulness of peanut agglutinin (PNA) for enriching subpopulations of thymocytes with different cell surface and functional phenotypes, we undertook a screening program to analyze forty other lectins for their binding to thymocyte and lymphocyte subpopulations. This study is now partially completed and demonstrated that a number of fluoresceinated lectins bind differently to thymocyte subsets as detected by FMF. The lectins which are primarily of interest are a series of plant lectins which bind galactose, N-acetyl-galactosamine or polymers of N-acetyl-glucosamine. The lectins binding to the cell surface is inhibitable, routinely, with the nominal sugar specificity. A number of lectins, particularly those binding galactose, have shown bimodal or trimodal fluorescent profiles on thymocytes which indicate a minor dull subpopulation and a major brighter subpopulation. For many of these lectins we have shown that the dull subpopulation is enriched in the cortisone resistant thymocyte (CRT) fraction obtained after *in vivo* cortisone treatment. This is in parallel to the enrichment seen for Thy 1 dull cells and an enrichment of Lyt 1^+23^- thymocytes. Using PNA to fractionate the cells we have further been able to enrich and recover both the dull population of many of these other lectins and the bright staining population which is lost in CRT. We are currently attempting to determine whether there are any discernable relationships between the carbohydrate binding sites of lectins and any of the anti-lymphocyte sera which we currently have available. In this regard, we have analyzed the dystrophic (*dy*) mutant mice which appear to have a pattern of thymocyte phenotype anomalies in some, but not all, of the "clinically" affected mice. Some of these mice appear to have a higher proportion of CRT-like cells by phenotypic analysis with antisera to Thy-1, Lyt 2, and AU-1 and a series of lectins including PNA. We have proposed that these mice may represent a continuum of affected disease states where the thymocytes of the most severely affected mice are more likely to express the cell surface phenotype of CRT.

4. Functional Studies of T-Cell Differentiation.

(a) Functional Aspects of Thymocyte Differentiation.

Selective agglutination by peanut lectin (PNA) has been shown to separate thymocytes (ThC) into defined subpopulations. It has been suggested that PNA agglutinable (PNA$^+$) ThC belong to an immature subpopulation, or to a second line of thymic differentiation. PNA nonagglutinable (PNA$^-$) ThC include the minority of all ThC, and although reported by some to be TL$^+$, seem to resemble cortisone-resistant ThC, as defined by both surface and functional criteria. We have shown that PNA$^-$ ThC interact with LNC to give synergistic responses in the MLR. Because *in vivo* synergistic responses have been attributed to the major TL$^+$ subpopulation of ThC (presumably PNA$^+$), it is important to note that the synergy *in vitro* seen here is found in the minor, PNA$^-$ subpopulation of ThC. Recently, analysis of thymocyte subpopulations by flow microfluorometry (FMF) has revealed the existence, among the PNA$^-$ fraction, of 3-5% Lyt-123$^+$ and 7% Lyt-1$^+$,23$^-$ subpopulations. It was of interest, therefore to determine whether these Lyt differentiated subpopulations represent mature thymocytes, ready for export, or ThC which must undergo further maturational step(s) before they can be classified as mature T-cells.

ThC separated into PNA$^+$ and PNA$^-$ subpopulations were tested alone and in titrated mixtures with LNC in the MLR. These same subpopulations were tested for their ability to respond to the T-cell mitogens, PHA and Con A. Using antibody-coated tissue culture plates (Mage plates), we are able to separate small numbers of PNA$^-$ ThC into Lyt-123$^+$ and Lyt-1$^+$,23$^-$ subpopulations, and test them in the above assays.

PNA$^-$ ThC are able to synergize with LNC in the MLR to provide greater than additive responses. When these ThC were separated into Lyt-1$^+$,23$^-$ and Lyt-123$^+$ subpopulations the following were found:

1. PNA-ThC separated into Lyt-1^{+}23^{-} and Lyt-123^{+} subpopulations by the Mage plate technique possess equal capacity to synergize with LNC in the MLR.

2. PNA^{-} Lyt-1^{+}23^{-} ThC are highly reactive to the T-cell mitogens, PHA and Con A, while PNA^{-}, Lyt-123^{+} ThC are completely unreactive to these mitogens at all doses tested.

3. Parallel studies using Lyt-1^{+},23^{-} and Lyt-123^{+} separated spleen cells show that both cell populations proliferate in response to low doses of Con A (1 g/ml) and PHA (5 g/ml), but that only the Lyt-1^{+},23^{-} subpopulations proliferate in response to high doses (10 g/ml, and 15 g/ml resp.).

4. These results suggest that PNA^{-}, Lyt-123^{+} ThC must undergo a further differentiation step before they are able to respond to low doses of PHA and Con A.

Future Course. We intend to examine further the functional differences between PNA^{-}, Lyt-1^{+},23^{-} and PNA^{-}, Lyt-123^{+} ThC and their peripheral counterparts in the spleen and lymph node. In addition, if the PNA^{-} Lyt-123^{+} ThC do represent nearly mature ThC which need to undergo further maturation before (or after) export to the periphery, what is the maturational influence?

(b) Role of External Antigens in Thymic Maturation.

It has been shown in *in vivo* transfer systems that ThC from tumor bearing mice suppress the transfer of tumor-immunity from sensitized mice to naive animals. We are investigating the ability to ThC from mice bearing the B-cell lymphoma, A-20 to interact with spleen cells in an MLR directed to A-20 antigens or to alloantigens.

Mixing experiments between ThC from mice bearing a footpad injection of A-20, versus normal ThC in the MLR were studied. In addition, FMF analysis on these same ThC using standard techniques have given the results described below.

Thymocytes from mice injected in the footpad one week previously with A-20 tumor cells show a marked increase in their ability to proliferate in response to alloantigens. These functional shifts are reflected by shifts in Thy-1.2 profiles analyzed by FMF. The Thy-1.2 profile is shifted toward an increase in dull staining cells (presumably more mature), but *no* shifts in Lyt-1.2 or Lyt-2.2 staining were observed. These results suggest that Thy-1 and Lyt antigens are acquired independently in the thymus, and may reflect differential maturation of a single subpopulation of ThC in response to injected tumor cells.

5. Analysis of T and B Cell Interactions with a Mixed Tumor, AKTB-1.

Another tumor model system which we have begun to use to analyze rare T and B cell subpopulation interactions is the AKTB-1 tumor. The AKTB-1 tumor line, previously described as having both T- and B-cell characteristics, was separated by electronic cell sorting (ECS) on a fluorescence-activated cell sorter (FACS) on the basis of surface immunoglobulin (sIg). This resulted in two distinct sIg$^+$ and sIg$^-$ subpopulations which were transferred in graded doses (10^1 to 10^3) into groups of normal AKR animals. The resulting splenic tumor cells were analyzed at several time points by flow microfluorometry for surface characteristics (sIg, Thy 1, Lyt 2, and Ly 9), and by erythrocyte-antibody rosetting for FcR. Two distinct sublines were identifiable - one of which was sIg$^-$, FcR$^-$, Thy 1.1$^+$, Lyt 2.1$^+$, PNA$^+$, Ly 9$^+$ and the other of which was sIg$^+$, FcR$^+$, Thy 1.1$^-$, Lyt 2.1$^-$, PNA$^-$, Ly 9$^+$. When these two sublines, generated by ECS fractionation and high dilution transfer, were serially transferred at high doses (10^5), they maintained their unique characteristics as distinct sublines, now designated AKTB-1t and AKTB-1b.

When these two cell lines were obtained, histopathology patterns were observed which reflected the cell surface phenotypes of the two component lines. One of these subsequently derived, in vivo tumor cell lines, AKTB-1b sIg$^+$) is distinguished by growth restricted to the spleen. Gross pathology indicates a nodular splenic growth with a deep red, highly erythrocytic component and virtually no tumor growth in lymph nodes or liver. The AKTB-1t (sIg$^-$) subline in contrast is distinguished by growth of a splenic tumor with a a smooth blanched appearance and tumor growth in virtually all lymphoid sites including liver, thymus and peripheral lymph nodes.

The difference in gross pathology of the sIg$^+$ and sIg$^-$ tumor cells is a reflection of the differential patterns of growth observed at the microscopic level where the sIg$^+$ subline grows in the follicular areas of the spleen and the sIg$^-$ subline grows as a diffuse tumor in the interfollicular areas and red pulp of the spleen.

It appears that the two tumors interact synergistically and that the B cell component may influence hematopoietic generation. This is under further investigation.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00137-14 LMI

PERIOD COVERED

October 1, 1970 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Biology of Graft-Versus-Host Reactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	Yvonne Rosenberg	Visiting Association	LMI/NIAID

COOPERATING UNITS (if any)

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Laboratory of Microbial Immunity

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Experimental Pathology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Induction of graft-versus-host reactions produces a transient nonspecific increase in numbers of immunoglobulin secreting cells of IgG1 and IgG2 type. Little increase in IgM or IgG3 synthesis was seen. (Work reported in Z01 AI 00134-18).

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00138-06 LMI

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Viruses and the Immune Response

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Herbert C. Morse III	Head, Virology & Cellular Immunology Section	LMI/NIAID
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	T. M. Chused	Senior Investigator	LMI/NIAID
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	E. Stockert*		
	B. A. Taylor**		
	R. A. Yetter	Guest Researcher (IPA)	LMI/NIAID
	P. Hoffman***		
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COOPERATING UNITS (if any) *Memorial Sloan Kettering, New York, New York
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.6	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Genes governing the expression of xenotropic murine leukemia viruses (X-MuLV) differ strikingly among inbred strains of mice. Two distinct loci affect the dominant expression of infectious viruses of NZB mice in crosses with other strains whereas expression of F/St infectious virus is markedly suppressed by a single locus in similar crosses. In addition, the tissue distribution of NZB infectious virus was shown to be bone marrow>spleen>thymus whereas in F/St mice infectious virus was found in thymus>marrow>spleen.

Expression of two cell surface antigens, XenCSA and G_{IX}, coded for by endogeneous X-MuLV, was shown to be influenced by different genes in crosses between C57BL/6 and DBA/2 or C3H mice: identical loci affect XenCSA levels in both crosses but different genes affect G_{IX} expression in the DBA/2 and C3H crosses. XenCSA expression on T cells is enhanced by treatment with PHA or Con A.

Finally, development of lymphoma and paralyses in NFS mice infected with wild mouse ecotropic MuLV was shown to be preceeded by the appearance of recombinant MuLV in splenic but not thymic tissues.

Project Description:

This project reflects longstanding collaborative studies with Drs. Hartley and Rowe of the Laboratory of Viral Diseases on the expression of MuLV in lymphoid tissues.

Expression of infectious X-MuLV. Previous studies documented that NZB mice have two loci determining the expression of infectious X-MuLV. In crosses with other inbred X-MuLV strains, NZB high expression of these two loci segregates as Mendelin dominants. By comparison, crosses of additional strains (F/St, BXD-14, NZW or M.M. molossinus) expressing moderate to high levels of infectious X-MuLV show that high expression is markedly suppressed in young F_1 mice and mice in the backcross to the low X-MuLV present. The possibility that the product of a single locus may suppress the high X-MuLV phenotype of non-NZB strains is under investigation.

Studies of expression of gp70 antigens coded for by X-MuLV. XenCSA and G_{IX} are two determinants of gp70 molecules coded for by endogenous X-MuLV. Studies of mice from crosses segregating for quantitative expression of XenCSA and G_{IX} confirmed previous work demonstrating that XenCSA levels are controlled by a gene linked to Gpd-1 on chromosome 4. For G_{IX}, a similar effect was observed for mice from one cross (B6 X DBA/2) but not another (B6 X C3H). These results indicate independent genetic control of two cell-surface markers for X-MuLV.

Other studies of spleen cells stimulated with PHA, Con A, or LPS showed that XenCSA expression is markedly increased by Con A and PHA but only sightly increased by LPS stimulation. Cell surface antigens coded for by X-MuLV may thus serve as markers for activation of T lymphocytes independent of increased production of infectious or X-MuLV.

Studies of neurotropic wild mouse ecotropic MuLV. NFS mice injected as newborns with CAS-BR-M, a wild mouse ecotropic MuLV, develop hind limb paralysis and lymphomas after 2-3 months. During this latent period, the level of XenCSA on spleen cells, but not thymocytes, increases markedly. This increase is associated with the appearance in spleen extracts, of antigens related to the gp70's of recombinant, mink cell focus-inducing (MCF) MuLV. MCF antigens were also detected in brains of older mice with paralysis. The possible role of recombinant MuLV in the development of paralysis is under investigation.

Resistance to MCF MuLV. Cells of DBA/2 and DBA/1 mice, as compared to those of NFS, AKR or some other inbred strains, are highly resistant to infection with MCF MuLV. Resistance is controlled by a single dominant gene. Studies are in progress to map this gene and determine its effect on spontaneous development of lymphomas.

Publications:

Elder, J. H., Gautsch, J. W., Jensen, F. C., Lerner, R. A., Chused, T. M., Morse, H. C., Hartley, J. W., and Rowe, W. P.: Differential expression of two distinct xenotorpic viruses in NZB mice. Clin. Immunol. and Immunopath. 15: 493-501, 1980.

Longstreth, J. D., and Morse, H.C. III.: Murine leukemia viruses of inbred mice, in press 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00139-15 LMI

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Immunologic Response of Animals to Trypanosomal Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. F. Finerty	Research Microbiologist	LMI/NIAID
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	L. P. Gasbarre*	Post-doctoral Fellow	
	L. Kendrick	Biologist	LMI/NIAID
	R. McKelvin**	Graduate Student	
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COOPERATING UNITS (if any)

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*** Medical College of Virginia, Richmond, Virginia

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Mice infected with various species of African trypanosomes, succumb 23-120 days after infection. Survival depends on the presence of IgM antibody. Mice with delayed IgM responses survive the longest. The data indicate a possible role of IgM antibody as a blocking agent. A new method was developed for assaying antibody responses to Trypanosoma rhodesiense.

PHS-6040
(Rev. 10-76)

Project Description:

The use of mice with naturally occurring immune deficiencies, particularly with regard to deficient humoral responses, should provide clues relevant to both host and parasite survival in animals infected with African trypanosomes. Since IgM synthesis figures prominently in host response to African trypanosomes, and was implicated in the non-protective host response to the parasite we chose CBA/N mice as the model for our studies. CBA/N mice possess a sex linked defect and are unable to synthesize IgM antibody to certain T-independent antigens. In addition, nude mice are used since IgM synthesis, in the absence of T_H induced IgG responses, can be assessed in terms of specific IgM antibody directed against the trypanosomes.

CBA/N mice revealed Ig responses that were totally different than CBA/CaJ and nude mice. IgM levels increased rapidly, peaked on day ten, decreased slightly and increased again on day sixteen. The IgM levels fluctuated, yet remained high throughout the infection. IgG2a levels showed a similar pattern to IgM. However, no IgG1 responses were noted in CBA/N mice during the period of study. Reverse PFC assays confirmed these findings. Whereas high numbers of B-cells secreting IgM and IgG2a were observed, only a slight increase in IgG1 secreting cells were detected. This result suggests that CBA/N mice had a class-restricted suppressor cell that was operating during infection. Another suggestion is that CBA/N mice lack a recognition signal for IgG1 responses to surface determinants on T. rhodesiense. No bromelain PFC were detected in CBA/N mice, until shortly before they died (days 58-65).

Taken together, these results suggest that T-suppressor cells were operative in CBA/CaJ mice, suppressing the Ig response, and prevented further expansion of B-cell clones in the presence of continuous trypanosome stimulation. However, the continual presence of an autoimmune IgM response (bromelain PFC's) in CBA/CaJ mice, and the lack of these cells in CBA/N mice suggest that CBA/N possess a suppressor cell for this type of response, or these mice lack the necessary B-cell clone to give this response. The suppressor cell hypothesis is favored, because bromelain PFC's were detected shortly before death, indicating the suppressive mechanism had broken down.

Both IgM and IgG antibody were detected simultaneously in T. rhodesiense infected CBA/CaJ mice, during the first ten days after infection. However, only IgG antibody was detected during the same period in CBA/N mice.

These results indicate that CBA/CaJ and CBA/N mice respond differently to T. rhodesiense stimulation. The presence of both IgM and IgG antibodies in CBA/CaJ mice suggest blocking of antigenic determinants on the trypanosomes. This resulted in detrimental effects on feedback mechanisms controlling the second wave of trypanosomes, leading to loss of IgM antibody, and rapid increase in parasitemia. CBA/N mice were not confronted with blocking of determinants, because only IgG antibody was present during the 14 days following infection. The data indicate that CBA/N mice possessed two types of IgM B-cell clones: (1) a clone that responded to polyclonal activation and no IgM antibody, and (2) another clone that required a different recognition signal and/or required two weeks for expansion before IgM antibody was synthesized.

During the course of these studies, IgG3 was detected by gel diffusion, in the sera of infected CBA/CaJ and CBA/N mice. Subsequent RIA studies showed that IgG3 serum levels increased rapidly in CBA/CaJ and CBA/N mice. Peak levels occurred on days eight and ten for CBA/CaJ and CBA/N mice respectively. These levels remained elevated throughout the infection. IgG3 levels also increased in nude mice. Further studies with nude mice revealed that only when IgM increased was there an increase in IgG3. These results indicate that IgG3 responses are T-independent. The data suggest that IgM and IgG3 may be controlled by the same regulatory mechanism.

Agglutination Method for Assaying T. Rhodesiense Antibody. Currently, two methods are used for assaying specific trypanosomal antibody, IFA and agglutination with live trypanosomes. The IFA is laborious for assaying large numbers of sera. The use of live trypanosomes presents a health hazard, particularly when human species are used. Plus, agglutination with live trypanosomes demands that a constant supply of infected animals always be available, and results are determined by microscopic examination. The method developed here utilizes trypanosomes killed with a low concentration of formaldehyde. To make the antigen visible, a blue vital stain is added, which is readily taken up by the dead trypanosomes. Antibody was assayed by using the microtiter system. Results demonstrated that specific trypanosomal antibody can be detected in mice infected with T. rhodesiense. This method allows for the first time, a simple, sensitive and reproducable procedure for assaying specific immune responses to African trypanosomes.

Publications:

Finerty, J. F.: African Trypanosomiasis: Cell-mediated and humoral immunity, in Proceedings of "Symposijm on Clinical Parasitology: Current Concepts in a Chaning Science, American Society of Microbiology, Philadelphia, PA, 1979, in press.

Finerty, J. F., Kendrick, L. P. and McKelvin, R. L.: B-cell activation in congenitally athymic and euthymic-mice infected with Trypanosoma rhodesiense. In Reed, N. (Ed.): Proceedings of the Third International Workshop on Nude Mice, in press, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00140-15

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Immunology of Malaria

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. F. Finerty	Research Microbiologist	LMI/NIAID
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	R. McKelvin*	Graduate Student	
	W. P. Weidanz**	Professor	
	D. Roberts***	Research Microbiologist	

COOPERATING UNITS (if any)
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**The Hahnemann Medical College, Philadelphia, PA
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LAB/BRANCH
Laboratory of Microbial Immunity

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Microbiology and Immunology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Non-lethal malaria was injected into macrophage deficient and normal mice. Resistance was noted in mice, whereas normal mice died. Results indicate that resistance was correlated to macrophage function and T-cell activity.

PHS-6040
(Rev. 10-76)

Project Description:

Phagocytic cells play a major role in the elimination of malarial parasites from the blood. Accordingly, we assayed C3H/HeJ and C57BL/10ScCR mice for their ability to clear non-lethal P. yoelii from their peripheral circulation. Macrophages from these mice do not shown in vitro cytotoxic properties. Normal C3H/HeN and C57BL/10ScN mice served as controls. Both C57BL/10 strains of mice cleared their parasites. C3H/HeJ mice also cleared their parasites although parasitemia was higher than C57BL/10 mice. However, C3H/HeN mice revealed a rapid rise in parasitemia and 75% of these mice succumbed to infection.

Since it is known the P. yoelii can revert to a lethal mutant when passaged in inbred mice, parasites were subinoculated from C3H/HeN with high parasitemia to NIH white swiss mice - the standard mouse strain for passaging this parasite. No increase in virulence was noted, the parasites retained the normal morphology and these mice recovered. Thus, the increase in parasitemia observed in C3H/HeN mice was the result of inherent properties of the C3H/HeN. These results indicate a gene function, previously unreported, associated with LPS resistance/sensitivity, but separate from these functions, yet apparently regulates macrophage activity in parasite clearance from the peripheral circulation. The ability of C3H/HeJ and C57BL/10ScCR mice to clear their infection is interesting in light of the fact that macrophages from these mice are unable to exhibit cytotoxic properties. Our results suggest that a signal(s) initiated by parasite stimulation of T-cells, enabled the macrophages to function in cooperation with other components of the immune system in removing the parasites. The inability of C3H/HeN to remove the P. yoelii suggests that these mice lacked the appropriate signal or are extremely sensitive to the endotoxin substances produced by malarial parasites, and may serve as a model for this type of assay.

Our studies with mice infected with African trypanasomes, revealed rapid increases in serum IgG3 levels shortly after infection. These parasites are extracellular blood parasites, whereas malarial parasites are intracellular (intraerythocytic). The question arose whether IgG3 responses were limited solely to extracellular parasites or whether intracellular malarial parasites could elicit IgG3 responses as well. Accordingly, CBA/N and CBA/CaJ mice were inoculated with lethal or non-lethal rodent malarial parasites. Serum was collected at vrious times after infection and IgG3 levels assayed by radioimmunoassay techniques. The results indicated that IgG3 increased rapidly in both CBA/N and CBA/CaJ mice within six days post infection. Mice infected with non-lethal malaria had slightly lowered IgG3 serum levels than did mice infected with lethal malaria. Mice infected with lethal malaria succumbed 8-10 days after infection and at this time, IgG3 levels were decreasing. By day 13, the IgG3 serum levels in mice with non-lethal malaria were >3000 µg/ml, the levels reamined elevated throughout their course of infection, and these mice survived. No differences were noted between CBA/N and CBA/CaJ mice. Thus, these results prove that intracellular malarial parasites can stimulate IgG3 responses in mice.

Publications:

Finerty, J. F., Kendrick, L. P. and McKelvin, R.: Chemical Enhancement of Protective Immunity. In Proceedings of a Food and Drug Administration Conference, *Inadvertant Modification of the Immune Response*, Washington, D.C., in press, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00141-06 LMI

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Immune Responses to Malaria and Related Intracellular Protozoa

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	K. J. Kim	Visiting Associate	LMI/NIAID
	F. M. Rollwagen	NIH Post-doctoral Fellow	LMI/NIAID
	C. B. Evans	Biological Lab Tech	LMI/NIAID
	J. Rodrigues-Ramos	Jr. Costep Officer	LMI/NIAID

COOPERATING UNITS (if any)

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.7	2.7	1.0

CHECK APPROPRIATE BOX(ES)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

During malaria infection the host responds to a variety of parasite Ags, but the location, number and specificity of the Ags involved have been difficult to determine. Thus during the last year we have used monoclonal antibodies (Abs) to study the antigenic nature of the rodent malaria parasite *P. yoelii*. The 18 hybridomas which have been studied in detail were produced by fusing P3-X63-NS/1 myeloma cells with spleen cells from mice infected for 4 or 6 days with the lethal or nonlethal strain of *P. yoelii*. Results show that the host responds to a number of Ags located either on the surface or within the cytoplasm of the parasite. The majority of monoclonal Abs identify Ags possessed by all erythrocytic stages of the parasite, but three bind preferentially to merozoites, and one is completely stage-specific for merozoites. Two of the 18 monoclonal Abs detect Ags shared by the sporozoite and erythrocytic stages of *P. yoelii*. Approximately 45% of the Abs detect species-specific Ags; 10% denote Ags confined to related rodent malaria parasites (*P. berghei*, *P. vinckei*, *P. chabaudi*); whereas, 45% detect Ags shared among rodent, primate (*P. knowlesi*, *P. falciparum*) and avian (*P. gallinaceum*) malarias. The 18 hybrids identify a minimum of 12 distinct plasmodial Ags. 24-35

Project Description:

During malarial infection the host responds to a variety of parasite antigens (Ag), but the location, number, and specificity of the Ags involved have been difficult to determine. Initially a biochemical approach was undertaken to identify the number of polypeptides present in extracts of the rodent malarial parasite *Plasmodium yoelii*. Two-dimensional analysis of parasite proteins using isoelectric focusing in the first dimension and SDS-polyacrylamide gel electrophoresis in the second, identified a minimum of 125 polypeptides. The separation and immunologic analysis of 125 polypeptides, using conventional biochemical methods, would be time consuming and probably require a prohibitively vast amount of starting meterial. Thus we decided to continue making use of hybridoma technology to study the immunologic nature of malaria parasites. Monoclonal antibodies (Abs) have provided us not only with reagents for isolating plasmodial Ags but also have allowed us to identify by immunofluorescence: (1) the location of Ags, i.e., Ags within or on the surface of parasites; (2) stage specific Ags, i.e., Ags unique to rings, trophozoites, schizonts and merozoites; (3) species specific Ags and (4) those Ags which are common to various members of the *Plasmodial* spp. i.e., crossreactive Ags. In addition, monoclonal Abs have been used to isolate malarial Ags. These Ags have then been tested in *in vitro* proliferation assays for effectiveness in stimulating various lymphocyte subsets from sensitized and normal animals.

Production of Anti-Plasmodial Secreting Hybridomas:

BALB/c mice were primed to malarial Ags in the following ways: (1) infected with 10^6 17XL parasites 4 days prior to fusion; (2) infected with 10^6 17XNL parasites 4, 6, and 11 days prior to fusion; (3) infected with the 17XNL strains, allowed to recover, and then inoculated with 17XL parasites 4 days prior to fusion (hyperimmune); (4) inoculated intravenously once or 3 times with irradiated *P. yoelii* sporozoites, and (5) vaccinated twice with an Ag preparation enriched for merozoites. Also BALB/c mice made congenitally athymic (BALB/c·Nu) infected with 17XL malaria 4 days earlier were used.

Fusions were performed using the procedure of Margulies, et. al., 1976 Cold Spring Harbor Symposium on Quantatative Biology 91:781. In brief, BALB/c spleen cells were fused with the P3-X63-NS/1 cell line using polyethylene glycol. Cells were distributed to microtiter wells and cultured in hypoxanthine-aminopterin-thymidine selection medium for 14 days. Culture supernatants were screened for anti-plasmodial activity using a solid-phase radioimmunoassay (RIA) developed by Kim, Taylor, Evans and Asofsky (manuscript submitted). RIA positive wells were verified by the indirect fluorescent antibody (IFA) technique. Hybrids, cloned by limiting dilution on a thymocyte feeder layer, were maintained *in vivo* in BALB/c mice.

Although positive hybrids have been produced using spleen cells from all primed animals, only those produced in fusions using spleen cells from mice infected 4 days with the 17XL and 4 and 6 days with 17XNL have been studied in detail. To date approximately 18 cell lines have been established. Hybrids produced in more recent fusions are in various stages of cloning and study.

The isotype of the anti-malarial hybrid cell lines was determined by Ouchterlony analysis of culture supernatants using class specific reagents, by immunoelectrophoresis (IEP) of ascites fluid using the above reagents, and by IFA analysis using fluoresceinated heavy chain specific goat anti-mouse IgM, IgG_1, and IgG_2. Monoclonal Abs of IgM, γ2a and γ3 classes have been found.

Ammonium-sulfate precipitated globulins from ascites fluids were used in most of the studies. However, some of the results have been confirmed recently using monoclonal Abs purified by protein A chromatography.

Identification of Stage Specific, Species Specific and Serologic Crossreactive Ags Using Monoclonal Abs:

At least 4 distinct patterns of fluorescence are produced by monoclonal Abs on fixed smears of P. yoelii. The patterns we have observed are similar, though not identical, to those reported by Freeman, et. al. (Nature 284: 366, 1980). Abs from 11/18 hybrid lines produce a fluorescence pattern identical to that observed using hyperimmune serum, 3/18 bind to Ags located in the membrane of either the parasite or infected RBC, 3/18 bind to all erythrocytic stages but when titered to 100 μg/ml bind only to a specific region within merozoites, and 1/18 is stage specific for merozoites.

The location of these Ags was next examined using IFA. One of the monoclonal (4L-7) binds to an Ag on the surface of freed parasites (most likely the parasitophorous vacuolar membrane). However, the remaining hybrids recognize Ags located in the interior of the parasite either associated with an organelle or cytoplasmic components.

The technique of IFA was used to determine if the hybrid cell lines secreted Abs against species specific or serologic crossreactive Ags, as numerous studies have demonstrated crossreactivity between members of the class Sporozoa. 45% of the hybrids are species specific for P. yoelii, 10% bind to Ags confined to rodent malaria parasites (P. berghei, P. vinckei, and P. chabaudi), but 45% show extensive crossreactivity among rodent, primate (P. knowlesi and P. falciparum) and avian (P. gallinaceum) parasites. Most of those in the last group are crossreactive with Babesia microti.

Based on the results from the above study, the 18 hybrids recognize a minimum of 12 Ags. It has not been possible to identify the number further using simple immunochemical means (PEG Ouchterlony, IEP, and crossed immunoelectrophoresis) as precipitin arcs do not form. Thus, the techinque of immune precipitation has been employed to characterize Ags of P. yoelii further. Extracts of P. yoelii were radioiodinated using the techniques of chloramine-T, Bolten-Hunter, and lactoperoxidase. Aliquots of labelled Ag were incubated with monoclonal Ab (ammonium sulfate precipitated from ascites or purified by protein A chromatography) and then absorbed on *S. aureus* Cowan strain or protein A coupled sepharose. Labelled-Ags were analyzed using SDS-PAGE and autoradiography. A problem of nonspecific binding of *Plasmodial* Ags to protein A has been encountered. Hopefully this technical difficulty can be circumvented in the coming year by using a second Ab (goat anti-mouse immunoglobuin) for immune precipitation instead of protein A.

Immunologic Studies Using Monoclonal Abs:

Ab has been shown to have a protective role in malaria as demonstrated by passive Ab studies. Recently Freeman, et. al. demonstrated that hybridoma-derived Ab specific for the merozoite stage could passively protect mice from an otherwise lethal infection. Ab from many of our hybrids have been passively transferred either alone or in combination to mice. Only one (6NL-10) altered the course of 17XL *P. yoelii* infection. This Ab is specific for merozoites. *P. yoelii*-infected BALB/c mice (0.1% parasitemia), treated with Ab from hybrid 6NL-10, showed significantly lower parasitemias than controls for 3 days following passive Ab treatment. The animals also survived 3 times longer than the controls. These results suggest that Abs from hybrid 6NL-10 have some role in protective immunity.

One aim of the project was to isolate Ags using monoclonal Abs and then to evaluate the immune response to these Ags using an Ag-induced proliferation assay. Recently, Sepharose 4B columns coupled with sulfate precipitated or purified monoclonal Abs have been prepared. Ags, eluted from these columns, have been added to *in vitro* cultures of normal and sensitized splenocytes and splenic subpopulations enriched for B, T, $Ly2^+$, and $Ly2^-$ cells. Of the 5 Ags studied, 4 appear to stimulate only sensitized lymphocytes, while the remaining one induces both normal and sensitized cells to proliferate. One Ag seems to stimulate primarily cultures enriched for sensitized B cells, whereas several other Ags stimulate $Ly2^-$ T cells to transform.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00142-07 LMI

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Development of Thymus-Derived (T) Suppressor and Amplifier Cell Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

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Laboratory of Microbial Immunity

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INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Inactive during current year.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00143-11 LMI

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Genetic Control of the Antibody Response to Type III Pneumococcal Polysaccharide (SSS-III)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. J. Baker	Head, Microbiology & Immunology Section	LMI/NIAID
OTHER:	B. Prescott*		
	G. Caldes	Chemist	LMI/NIAID
	D. F. Amsbaugh	Biologist	LMI/NIAID
	P. W. Stashak	Microbiologist	LMI/NIAID
	D. W. Bailey**		

COOPERATING UNITS (if any)
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LAB/BRANCH
Laboratory of Microbial Immunity

SECTION
Microbiology and Immunology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.3	0.7

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The results of detailed genetic studies with Bailey recombinant-inbred (RI) strains of mice have shown that multiple genes, acting in a complementary and independent manner, influence the capacity of mice to give an antibody response to Type III pneumococcal polysaccharide (SSS-III). The high (near 50%) frequency of unique or recombinant phenotypes observed suggests that these genes may be located on different chromosomes. Support for this view was derived from studies using other RI strains. The genes involved, which tentatively have been assigned to chromosomes 17, 9 and 4, as well as the H-8, H-17, H-20, and H-28 loci, appear to be specific for the antibody response to SSS-III.

PHS-6040
(Rev. 10-76)

Project Description:

The availability of recombinant-inbred (RI) strains of mice, as well as experimental approaches which enable one to examine separately the activities of B cells, suppressor T cells and amplifier T cells, provides an excellent means to obtain information on the genetic mechanisms involved in the expression of these cellular processes as they apply to the antibody response to SSS-III. The results of detailed genetic studies with Bailey RI strains of mice have shown that multiple genes, acting in an independent and complementary manner, influence the expression of each of these functional activities; none of the genes involved are linked to the H-2 or the IgC_H allotype complexes. In fact, the rather high (near 50%) frequency of unique or recombinant phenotypes observed suggests that these genes may be located on different chromosomes.

Bailey RI strains of mice are derived from BALB/cByJ and C57BL/6ByJ progenitor strains which differ greatly in their capacity to make an antibody response to SSS-III; BALB/cBYJ mice are high responders, whereas C57BL/6ByJ mice are low responders. With the availability of 23 different B6·C congenic strains bearing known chromosomal segments from BALB/cByJ mice on a C57BL/6ByJ background, it is possible to assign some of the immune response genes for the antibody response to SSS-III to particular chromosomes or linkage groups. Here, one would simply immunize mice with an optimally immunogenic dose of SSS-III and then look for antibody responses, significantly greater than that of the low responding background strain; higher responses would indicate contributions made by genes originating from high responding BALB/cByJ mice and establish their location on individual chromosomes. The results of current studies with B6·C congenic strains indicate that multiple genes which influence the capacity of B cells to respond to SSS-III may well be on different chromosomes. Tentatively, they appear to be associated with chromosomes 17 (probably not H-2 linked), 9 and 4, as well as the H-8, H-17, H-20, and H-28 loci which have not yet been assigned to any particular chromosome(s). Furthermore, these immune response genes seem to be specific for the antibody response to SSS-III since they do not influence responsiveness to α 1-3 dextran, an unrelated antigen. At present, we are attempting to determine whether environmental and/or regulatory T cell factors have more - or less - of an influence in governing the expression of some of these immune response genes. Also, studies on the idiotypic repertoire of antibodies specific for SSS-III (now in progress) should provide information on whether they represent structural genes for antibody or multiple copies of the same gene that has been translocated to different chromosomes by a random process.

During the course of other unpublished studies on the non-adjuvant-induced generation of carrier-specific helper T cell activity, we noted that the expression of this activity in C57BL/6ByJ mice is extremely low, whereas it is relatively high in BALB/cByJ mice for at least one type of carrier. A more detailed examination of the genetic basis for this difference is underway using Bailey RI strains of mice. The results of this study should (a) provide further information on whether the activities of amplifier T cells, which have been examined previously and helper T cells are under separate genetic control and (b) reveal additional contrasting differences between these two cellular activities.

Publications:

Baker, P. J., Amsbaugh, D. F., Stashak, P. W., Caldes, G., and Prescott, B.: Immunological characteristics and central mechanisms involved in the antibody response to Type III pneumococcal polysaccharide. Revs. Infectious Diseases, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00144-16 LMI

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Regulation of the Antibody Response to Pneumococcal and Other Polysaccharide Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. J. Baker	Head, Microbiology & Immunology Section	LMI/NIAID
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OTHER:	K. R. Schroer	Research Associate	LMI/NIAID
	B. Prescott*		
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COOPERATING UNITS (if any) *Biolgoical Research Institute, Rockville, Maryland 20852
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LAB/BRANCH
Laboratory of Microbial Immunity

SECTION
Microbiology and Immunology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.8	0.7

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Oscillations in the production of antibody secreting cells has been noted after immunization with bacterial lipopolysaccharide and levan. Such oscillations may be the result of negative feedback mechanisms exerted by thymus-derived suppressor cells or by auto-antiidiotypic antibodies.

PHS-6040
(Rev. 10-76)

Project Description:

One aspect of this work is to understand the factors responsible for the generation of oscillations in the production of antibody secreting cells. Two different antigens are used in this investigation: lipopolysaccharide (LPS) from E. Coli 113 and bacterial levan (BL) from Aerobacter laevinicum.

We have observed oscillations in the number of anti-LPS PFC from the spleen of various strains of mice (BALB/c; C3H/HeN; C3H/HeJ; (BALB/c X C3H/HeN)F_1; NIH Swiss mice; NIH nude mice on Swiss background; and, C57BL/6N) and have investigated the contribution of various factors to the generation of these oscillations. Theoretical studies indicate that oscillations generally result from the existence of negative feedback mechanisms. The latter might be due to (a) a negative control exerted by T suppressor cells, or (b) a negative control exerted by auto-anti-idiotypic antibodies. Oscillations might also result from successive waves of antigenic stimulation, if the LPS remained in the spleen for a long time. LPS is composed of a polysaccharide moiety which is responsible for its antigenicity, and of lipid A, which contributes to its toxicity. We have examined whether toxicity plays a role in the generation of oscillations by comparing the PFC response of C3H/HeN and C3H/HeJ mice to an optimal dose of LPS (20 µg). C3H/HeJ mice do not support an in vitro mitogenic response to LPS (2) and are resistant to the toxic effects of high concentration of LPS; this indicates that they have no receptors for lipid A. The results of our study showed that both strains give an oscillatory response although the magnitude of the response of C3H/HeJ mice was lower.

An experiment of Britton seems to indicate that the immunogenecity of LPS persists in the spleen for a long time. To investigate this aspect, we have examined the clearance pattern of the spleen after the injection of 20 µg of LPS together with a small quantity of radioactive ^{125}I-LPS. The latter had been prepared by Dr. Rudbach according to the method of Ulevitch. This study indicates that the quantity of LPS persisting in the spleen for two weeks is of the order of 0.030 µg. Therefore, it can be conjectured that the antigen persists in the spleen and is able to restimulate the differentiation of antibody secreting cells.

To investigate the role of T cells in the response to LPS, we have compared the immune response in NIH Swiss mice and in NIH nude mice on Swiss background. Both responses are comparable and present a cyclical character. Unfortunately, these mice belong to outbred strains which precludes more detailed analysis. Therefore, we intend to study the responses of BALB/c nu/nu to LPS.

In the case of BALB/c mice, it appears that the amplitude of the oscillations drops considerably after 18 days. We have tested if this drop is due to a shift from IgM to IgG production. IgG, which has a slow metabolic rate, could exert antibody-mediated feedback inhibition. However, we have failed to detect indirect (IgG producing) PFC. Preliminary experiments indicate that spleen cells as well as serum from mice immunized 35 to 40 days earlier can suppress the peak of the immune response to LPS if injected together with antigen. We intend to study the origin of this suppressive mechanism in greater detail.

First of all, we will investigate if T cells are involved in this suppression. For this, we will enrich T cells by passing spleen cells suspension over a nylon wool column. We also intend to separate the various subpopulations of T cells by absorption on plates coated with specific monoclonal antisera. On the other hand, since the serum can also inhibit the first peak of the oscillation, we are planning to characterize the serum component which produces this effect. Various studies indicate that putative auto-antiidiotypic antibodies appearing late in the course of the immune response can suppress the primary response in other mice after passive transfer. Therefore, we would like to establish if the suppressive factor found in the serum 35 to 40 days after the antigenic challenge is antiidiotypic antibody. In particular, we must exclude the existence of an antibody mediated feedback inhibition. For this, we will absorb the serum on an LPS affinity column to remove anti-LPS antibody and determine if the suppressive capacity remains. To establish the antibody nature of this suppressive factor, we will check if the activity of the serum component is abrogated by absorption on a normal mouse serum affinity column or by absorption with anti-mouse immunoglobulin reagent.

Another way of detecting the role of autoantiidiotypic antibodies would be to investigate if PFC against erythrocytes coated with anti-LPS antibodies appear in the course of the immune response. For that, we need fair amounts of anti-LPS antibody and we intend to develop anti-LPS hybridomas in collaboration with Dr. Schroer. If we get monoclonal anti-LPS antibodies, we are also planning to produce antiidiotypic serum in BALB/c mice and in rabbits. This should allow us to investigate if there is a variation in the expression of idiotypes in the course of the oscillatory phase of the immune response. We think that this study should allow us to determine if the production of auto-antiidiotypic antibodies regulate the oscillatory behavior of this immune response as in the case of the response to phosphorylcholine.

We have observed that the number of PFC also oscillates during the response to BL in the case of BALB/c mice. We intend to study the role of idiotypic regulations in this system. Indeed, we have been able to detect auto-anti-idiotypic PFC against erythrocytes coated with the bacterial levan binding myeloma protein EPC 109. EPC 109 and anti-levan antibody share the cross-reactive idiotypes Id X G, Id X E, Id X D, IdC and Id X A. Sixty percent of the anti-BL PFC express the E 109 idiotype at the beginning of the immune response. This percentage diminishes afterwards. We would like to investigate the role of anti E 109 antibody in the regulation of the oscillatory pattern and in the modulation of the expression of the E 109 idiotype.

Publications:

Hiernaux, J.: Network regulatory mechanisms of the immune response. In Bona, C., and Cazenave, P.A. (Eds.): Regulation of Function of Lymphocytes by Antibodies. New York, Wiley, 1980, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00145-13 LMI

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Mode of Action of Thymus-Derived (T) Suppressor and Amplifier Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P.J. Baker	Head, Microbiology & Immunology Section	LMI/NIAID
OTHER:	K.B. Schroer	Research Associate	LMI/NIAID
	P.W. Stashak	Microbiologist	LMI/NIAID
	D.F. Amsbaugh	Biologist	LMI/NIAID
	G. Caldes	Chemist	LMI/NIAID
	B. Prescott*		
	K. J. Kim	Visiting Associate	LMI/NIAID
	J. Winkelstein**		
	D. Briles***		

COOPERATING UNITS (if any)

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**Johns Hopkins University, Baltimore, Maryland
***University of Alabama Medical School, Birmingham, Alabama

LAB/BRANCH

Laboratory of Microbial Immunity

SECTION

Microbiology and Immunology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.7	0.8

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Hybridomas making antibodies specific for Type III pneumococcal polysaccharide were used to investigate a variety of issues associated with regulation of the immune response to this antigen. Such antibodies were capable of confering protection against pneumococcal challenge and possessed an idiotypic pattern found in rather low frequency in the serum of mice immunized with SSS-III.

PHS-6040
(Rev. 10-76)

Project Description:

Four areas of investigation dealing with regulation of the antibody response to Type III pneumococcal polysaccharide (SSS-III) are currently underway using hybridoma anti-SSS-III products (HP). The first area involves the diversity of the anti-SSS-III BALB/c antibody response.

1. This project was initiated in January 1978, in order to obtain a basic immunochemical characterization of the diversity of anti-SSS-III antibody responses, so that the role of amplifier and suppressor T cells could be assessed in greater detail. At that time the isotypic profile of anti-SSS-III PFC was known but no examination of V-region diversity had been performed. This issue of diversity is important from a standpoint of interpreting various models of suppressor and amplifier T-cell activity upon B cell target populations of anti-SSS-III committed lymphocytes (1). If anti-idiotypic models of cellular interaction are correct, then the size of the repertoire of responding B cells would dictate the diversity of controlling T cell populations as well. This would add to our understanding of molecular requirements of T cell regulated suppression.

Since the response to SSS-III is largely IgM, isoelectric focusing was found to be unsuitable for examining spectrotypic diversity. Thus, an attempt to dissect the diversity problem was made by generating hybridomas which would secrete large quantities of monoclonal antibodies whose idiotypic characteristics might be examined immunochemically.

Initially, these hybridomas were generated with the 45.6TG1.7 subline of the in vitro thioguanine resistant myeloma cell line of MPC 11 (an IgG2b, κ secretor). These hybridomas were purified by affinity chromatography over SSS-III-aminoethyl-sepharose 4B columns and found to be highly pure preparations of IgA, κ and IgM, κ homogeneous antibodies. Spectrotypic analysis, by isoelectric focusing, of the isolated purified heavy and light chains of these proteins revealed - highly conserved patterns of light chain expression (only two patterns were found), but additionally revealed that some hybrid antibody molecular assembly was occurring with the parental myeloma light chain, a Kappa chain with a distinct spectrotype. No mixed heavy chain assembly was seen.

These hybridoma products (HP) were used to prepare anti-idiotypic antisera in Guinea Pigs. Subsequently, a cross-reactive idiotype of one of five HP was found to be widely distributed among many strains of mice with diverse allotypic and H-2 genotypes (2).

The mere presence of determinants similar to the HP Id in the sera of immune mice suggests that the correct HL anti-SSS-III binding site is being recognized, rather than the anomolous $H_{SSS-III}$ $L_{MPC\ 11}$ site(s). Additionally, this idiotype reactivity is ligand modifiable by SSS-III.

Further studies to confirm these findings are underway with recombinant HL pairing experiments for idiotypic H x L chain assignments. Additionally, new hybridomas are being prepared with the SP2/0 non-secretor cell line of MOPC 21, which has now become available to us.

Overall, these experiments suggest an assignment of a small fraction of anti-SSS-III antibodies to a given idiotypic pattern. Many more hybridoma analyses will be required in order to draw conclusions about the extent of BALB/c anti-SSS-III V-region diversity for which models of cellular interactions must account.

2. A second area of interest entailed the use of somatic cell hybridization in an attempt to complement the defective B cell responses of CBA/N murine B cells to a TI-2 antigen (SSS-III). Spleen cells from defective CB (CBA/N x BALB/c F_1 males) mice were compared to their genetically similar, non-defective, BC (BALB/c x CBA/N F_1 male) counterparts for their ability to fuse with NS1 myeloma cells and to give rise to effective anti-SSS-III secretion products. Each parental type was able to produce γM specific anti-SSS-III antibodies, however, the isotype diversity was greater in the non-defective BC mice than in the CB mice bearing the X-linked defect .

3. The third area of investigation is collaboratively being performed at the University of Alabama by Dr. David Briles. These studies investigate the role of protective antibody in the host defenses of mice challenged with Type III pneumococci. Preliminary studies using hybridoma products have shown passive protection of (CBA/N x DBA/2)F_1 male mice when challenged by pneumococci. (These defective mice bear the X-linked genetic trait of antibody unresponsiveness to Type III pneumococci).

4. The final area of collaborative effort is conducted in the laboratory of J. Winkelstein, (Johns Hopkins University). He has previously demonstrated the activation of the alternate pathway of complement by pneumococci involves cell wall components. Current studies are utilizing specific IgM, κ anti-SSS-III capsular polysaccharide HP antibodies to localize the sites of action of antibodies in their anti bacterial effects.

Publications:

Schroer, K. R., Kim, J. K., Amsbaugh, D. F., Stashak, P. W. and Baker, P. J.: Lymphocyte hybridomas which secrete antibody to the Type III pneumococcal polysaccharide: idiotypic characterization. In, Schelssinger, D. (Ed.) Microbiology 1980, Washington, D. C., American Society for Microbiology, 1980, in press.

Schroer, K. R., Kim, J. K., Prescott, B., and Baker. P. J.: Generation of anti-type III pneumococcal hybridomas from mice with an X-linked B-lymphocyte defect. J. Exp. Med. 150: 698-702, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00146-07 LMI

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Immunological Studies on Components Isolated from Bacteria, Parasites and Plants.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. J. Baker	Head, Microbiology & Immunology Section	LMI/NIAID
OTHER:	B. Prescott*		
	G. Caldes	Chemist	LMI/NIAID
	D. F. Amsbaugh	Biologist	LMI/NIAID
	P. W. Stashak	Microbiologist	LMI/NIAID
	P. R. Beining**		
	G. M. Flannery**		
	P. Rebers***		

COOPERATING UNITS (if any)
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**Department of Biology, University of Scranton, Scranton, Pennsylvania 18510
***U.S. Department of Agriculture, Northern Regional Lab., Ames, Iowa

LAB/BRANCH
Laboratory of Microbial Immunity

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Microbiology and Immunology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.2	0.8

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The results of these investigations show that the immunogenicity of poorly immunogenic antigens such as the lipoteichoic acid of Staphylococcus aureus can be increased substantially by attachment to a carrier molecule capable of activating thymus-derived (T) helper cells. This approach is being examined to increase the immunogenicity of other microbial cell-surface antigens of biomedical importance.

Project Description:

One of the main objectives of this study is to develop approaches for improving the immunogenicity of poorly immunogenic microbial antigens of biomedical importance. Among the antigens considered are the membrane lipoteichoic acid (LTA) of Staphylococcus aureus, which may play an important role in the pathogenesis of staphylococcal infections, and the capsular polysaccharide of Type VI Streptococcus pneumoniae (SSS-VI), the bacterium which is the major cause of pneumococcal infections (otitis media) in children of pre-school age.

Since existing methods for detecting serum antibody specific for LTA were unreliable, a procedure first had to be developed for this purpose. Using a modification of the periodate-oxidation method of Sanderson and Wilson, we were able to couple LTA to indicator erythrocytes so that such cells could be used for the detection of serum antibody by both passive immune hemolysis and hemagglutination, as well as for the detection of antibody-producing plaque-forming cells (PFC). As a result, these immunological procedures were found to be quite sensitive for the detection of antibody specific for LTA; almost all of the antibody made is of the IgM class.

Although mice were found to make a significant LTA-specific serum antibody and PFC response after immunization with heat-killed S. aureus, virtually no antibody could be detected after immunization with isolated LTA, over a 10,000-fold dose range; this confirmed the fact that isolated LTA is poorly immunogenic. The ability of anti-lymphocyte serum (ALS) to abolish the antibody response produced after immunization with S. aureus, as well as the failure to obtain such a response in athymic nude mice suggested that LTA in association with a cell wall or cell membrane component capable of activating thymus-derived carrier-specific helper cells, is required for the development of an antibody response to LTA. This was confirmed by the results of subsequent studies in which it was found that non-immunogenic amounts of LTA bound to various carriers (sheep, turkey or horse erythrocytes) were able to elicit a substantial PFC and serum antibody response to LTA only in mice pretreated (primed) with the same carrier. It should also be noted that prior treatment with isolated LTA did not reduce the capacity of primed mice to respond to LTA bound to carrier; thus, the failure to respond to isolated LTA could not be attributed to its capacity to induce tolerance. Studies are now in progress to determine whether other carriers, more suitable for use in humans (e.g., tetanus toxoid), can be employed for this purpose. It is anticipated that the results of these investigations might ultimately be applied to the development of an effective vaccine to deal with antibiotic-resistant staphylococcal infections.

Type VI pneumococcal polysaccharide is one of the components of the Pneumovax vaccine which has been developed, under contracts supported by the NIAID, for the prevention of over 85% of the pneumococcal pneumonias in man. Adults have been found to give a reasonably good serum antibody response to SSS-VI;

however, SSS-VI is poorly immunogenic for children under two years of age. In this age group, a high percentage of pneumococcal otitis media is caused by Type VI S. pneumoniae and several antibiotic-resistant strains have been isolated. Obviously, there is a need to improve the immunogenicity of this antigen in order to improve the efficacy of the vaccine for children. Attempts to prepare indicator erythrocytes for the detection of antibody specific for SSS-VI using the periodate oxidation, chromium chloride or the cyanuric chloride methods were unsuccessful; this may have been due largely to the lack of charged groups on SSS-VI or the destruction of galactose residues which appear to be a major component of the immunodominant group. However, the preparation of a palmitoyl derivative of SSS-VI, by the palmitoyl chloride method, was found to give excellent results in this regard. Mice were found to give a reasonably good serum antibody and PFC response after immunization with heat-killed Type VI pneumococci; however, no serum antibody or PFC could be detected after immunization with purified SSS-VI obtained from different sources, regardless of the dose employed. The results of preliminary studies suggest that the attachment of SSS-VI to an appropriate carrier will greatly improve its immunogenicity, as was the case for LTA; studies are now in progress to establish optimal conditions for producing this effect with this antigen.

A substantial amount of information on the ontogeny of the antibody response to Type III pneumococcal polysaccharide (SSS-III) has been derived from previous studies. Mice do not make a detectable PFC response to SSS-III until two weeks of age; numbers of PFC comparable to those attained in adult mice are not attained until four weeks of age. Using the approaches described above, we will examine whether they would be of value in improving immunogenicity in mice, not yet capable of giving adult responses to isolated (purified) SSS-III and other type specific polysaccharides. A detailed analysis of the maturation of carrier-specific helper T cell activity will also be a necessary part of such a study.

Publications:

Beining, P. R., Flannery, G. M., Caldes, G., Prescott, B., and Baker, P.J.: Use of the periodate oxidation coupling method for the detection of antibody and antibody-producing cells specific for staphylococcal lipoteichoic aicd. J. Immunol. Meth. 32: 167-176, 1980.

Beining, P. R., Flannery, G. M., Prescott, B., and Baker, P. J.: Influence of carrier-specific thymus-derived cells on the IgM antibody response to staphylococcal lipoteichoic acid. Infection and Immunity, 29:132-139, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00153-04 LMI

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

In Vitro Response of Human Peripheral Lymphocytes to Infectious Organisms.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Richard Asofsky	Laboratory Chief	LMI/NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Microbial Immunity

SECTION

Experimental Pathology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

No progress was made in the past year.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00158-04 LMI

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Immune Response to Entamoeba Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. F. Finerty	Research Microbiologist	LMI/NIAID

COOPERATING UNITS (if any)

NONE

LAB/BRANCH

Laboratory of Microbial Immunity

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Microbiology and Immunology Section

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NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Inactive during current year.

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00186-07 LMI

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Pathogenesis of Autoimmunity in Inbred Strains of Mice

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. M. Chused	Senior Investigator	LMI/NIAID
OTHER:	H. C. Morse III	Senior Investigator	LMI/NIAID
	W. F. Davidson	Visiting Fellow	LMI/NIAID
	V. Manohar	Visiting Fellow	LMI/NIAID
	E. Brown	Biologist	LMI/NIAID

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Microbial Immunity

SECTION

Virology and Cellular Immunology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 2.0	PROFESSIONAL: 1.5	OTHER: 0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The immunologic mechanism and genetic control of autoimmune disease is being investigated in several autoimmune strains of mice utilizing flow microfluorometry and functional analysis of lymphocyte subpopulations.

Project Description:

The long-term goal of this Laboratory is to advance our understanding of the molecular basis of autoimmune disease so that (a) safer, more specific, therapy may be designed, and (b) our knowledge of immunoregulation will be enhanced. Several different strains of inbred mice which spontaneously develop autoimmune disease are the current objects of study in this project.

The New Zealand Black (NZB) mouse was the first autoimmune strain described. In recent years, we and other laboratories have shown that NZB mice have a primary B cell abnormality. Their spleen cells spontaneously secrete forty times as much pentameric IgM as spleen cells from normal mice; have ten times as many IgM containing plasma cells; and have ten times as many pfc to several common haptens. These abnormalities are detectable at birth and are present in nu/nu and T deprived NZB mice. These observations indicate that NZB mice develop T cell independent polyclonal activation of B cells. There are two additional facets to the abnormalities of NZB B cells. First, their spleens contain a large fraction of cells with reduced levels of surface IgD. Two-color immunofluorescence studies with the dual-laser fluorescence activated cell sorter shows that the δ-dull cells are μ-bright, and that NZB spleen contains a much greater fraction of such cells than normal mice. The IgM secreting cells are part of this subpopulation. Second, the plasmablasts found in NZB spleen contain and secrete four times as much IgM as those found in normal mice.

NZB mice also express a primary T cell abnormality. They exhibit a primary cytotoxic response in vitro to H-2 compatible mice. Mu-suppression experiments have shown this to be independent of the B cell activation.

We are now examining (NZB X B10.D2) X B10.D2 progeny for T and B abnormalities and have found (a) both the T and B parameters fail to show descrete segregation suggesting that more than one gene influences them, and (b) the T and B abnormalities segregate independently. Thus, we conclude that NZB mice have a surprisingly large number of genes that condition their basic immunologic abnormality. There are likely to be additional genes that control the specificity of autoantibody produced.

We had hoped to fully dissect the genetics of NZB autoimmune disease. The apparent complexity we have uncovered makes this a task of unappealing proportions. We do plan, however, to try to determine the relative importance of the T and B cell abnormalities to the development of autoimmune disease. Our main requirement at this point is an easily measurable correlate of in vivo T cell activation, preferably utilizing the FACS. We are investigating several sera (anti-ALA, Ly6, Ly8), membrane probes of charge and fluidity, and a panel of rat anti-NZB spleen hybridomas in this regard. We will then reexamine the progeny of NZB X B10.D2 backcrossed to both parental strains for associations between autoantibody production, the B cell abnormalities described above, and any T cell parameter which we are able to measure.

The genetics of some of the more recently described autoimmune strains appear to be more straightforward than NZB. We studied the single recessive me/me gene on the C57/BL6 background and concluded that it affected both T and B cell function. Similar studies using a panel of affinity purified and monoclonal antisera (anti-Thy 1.2, Ly1, Ly2, IgD, IgM, IgG1, IgG2) and the FACS on several autoimmune strains are now in progress. It is already clear, from our work and that of others, that each strain is phenotypically distinct, and will probably prove gentically distinct as well. This leads to the important conclusion that human patients with autoimmune disease can be expected to manifest at least as much genetic heterogeneity (the phenotypic heterogeneity is already apparent clinically) as the inbred mouse strains under study.

Our plan for the future is to select a limited number of autoimmune strains with the most restricted abnormalities and attempt to determine the pathologic mechaniam at the molecular level.

Publications:

Chused, T. M., H. M. Moutsopoulos, S. O. Sharrow, C. T. Hansen, and H. C. Morse III.: In, Genetic Control of Autoimmune Disease. N. L. Rose, P. E. Bigazzi, and N. L. Warner, Eds. Elsevier/North Holland, New York, p. 177, 1979.

Chused, T. M., H. M. Moutsopoulos, S. O. Sharrow, and C. T. Hansen.: In, B Lymphocytes in the Immune Response. M. Cooper, D. Mosier, I. Scher, and E. Vitetta, Eds. Elsevier/North Holland, New York, p. 363, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00187-07 LMI

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies in Sjogren's Syndrome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. M. Chused	Senior Investigator	LMI/NIAID
OTHER:	E. M. Brown	Biologist	LMI/NIAID
	H. Moutsopoulos	Visiting Scientist	LMI/NIDR
	T. Lawley	Senior Investigator	D/NCI
	D. Mann	Senior Investigator	I/NCI

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CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Inactive during current year

PHS-6040
(Rev. 10-76)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00131-13 LMI (formerly Z01 AI 00131-12 OSD)

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Mechanisms of Hypersensitivity in Inbred Histocompatible Guinea Pigs

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. H. Stone	Head, Experimental Autoimmunity Section	LMI/NIAID
OTHER:	C. S Raine*		
	R. B. Nussenblatt	Senior Investigator	CB/NEI

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NIAID, NIH, Bethesda, Maryland 20205

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1.0	1.0	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Chronic allergic encephalomyelitis (EAE) is induced in juvenile guinea pigs, but the adoptive transfer from juvenile animals yields only acute EAE in recipients. This adoptive EAE can be inhibited by injection of recipients with spinal cord in incomplete Freunds adjuvant (IFA), however, the opposite effect of transfer of the putative suppressor cells has not been shown. Thus, transfer of lymph node cells from donors receiving spinal cord in IFA does not protect recipients from actively induced acute or chronic EAE. Juvenile strain 13 guinea pigs can show an acute stage of EAE before the chronic illness, and it has been observed that they may show cataracts during this acute EAE. These cataracts were usually doughnut-shaped, with the opacity beginning at the cortex of the lens and filling in with time. Some of these remitted with the recovery of the young guinea pigs from acute EAE, and did not return with the consequent chronic disease.

PHS-6040
(Rev. 10-76)

Project Description:

Although it is doubtful whether the guinea pig may be useful in contributions to theoretical genetics in the future because of the shortages of genetic material, pure strains of cavia have and will become important in other disciplines. Inbred guinea pigs were available for genetic studies as early as inbred mice, but only two of the original strains have survived for the heavy duty reflecting current interest in immunogenetics. However, the two strains are different in several types of responses of interest to immunologists, and these differences have been investigated in our laboratory. For example, strain 2 is resistant to anaphylactic shock, and we have shown some years ago that this is due to a shortage of liberable histamine in the lungs. More recently, we have been investigating differences in susceptibility to experimental allergic encephalomyelitis (EAE) with the result that we have described a reproducible chronic form of EAE in strain 13 guinea pigs as an animal model most reminiscent of multiple sclerosis. We have proceeded to take advantage of the availability of histocompatible animals of good size which have retained their reputation of species of choice in areas of hypersensitivity and autoimmune disease research.

Adoptive Transfer of EAE from Immature Donors Susceptible to Chronic Disease

If strain 13 guinea pigs are injected when they are still immature (< 250 gms) with suspensions of spinal cord incorporated into complete Freund's adjuvants (CFA), they eventually (<2 mos later) develop a chronic EAE characterized by demyelination in the central nervous system (especially in the lumbar spinal cord) and paralysis of the rear legs with atrophy of the hind quarters. They show clinical and structural evidence of repetitive episodes of demyelination and remyelination. It was established some time ago that acute EAE may be transferred between histocompatible guinea pigs using living lymph node cells. Through application of the same techniques using juvenile strain 13's as donors, chronic EAE was not observed in recipients, most of which, juvenile or adult, developed severe acute EAE. Why the adoptively immunized strain 13 shows the acute disease rather than the chronic, is being studied. The following points may be relevant to this investigation: 1) In actively immunized guinea pigs, the sensitized T cells are in a circumscribed zone of the draining lymph nodes. It is possible that suppressor T cells under these circumstances are in direct contact with the "cytotoxic" T cells sensitizable to CNS antigen, and since suppressor cells have been reported to be more active in younger animals they could thus modulate an autoimmune disease. When cell suspensions are injected into recipients, however, each cell migrates on its own and might be under minimal influence by putative suppressor cells. 2) In the juvenile chronic model, relative differences in susceptibility might also lie in the latest stages of the pathogenesis, with younger animals (which show the histological signs of mononuclear cell cuffing in the CNS whether clinically affected or not) producing less of a cytotoxic factor acting on nerve cells during an acute stage in EAE. Thus, the immature animals survive to go on to a chronic state, but they have always had lymphocytes capable of inducing acute EAE.

Future plans involve evaluation of physically or immunologically sorted subsets of lymphocytes in recipients. From another viewpoint, the use in transfers of node slices instead of suspensions will test the role of lymphoid architecture. We have already shown slices suitable for transfer of sensitivity to simple chemicals and proteins.

Immune Deviation and Other Suppression of Acute and Chronic EAE

Some years ago M. Kies and her colleagues suppressed the acute EAE by prior injection of the myelin basic protein in incomplete Freund's adjuvant (IFA) and also by injection of CFA alone before the sensitizing CNS antigen in CFA. We had worked with Geoffrey Asherson on the mechanisms of "immune deviation" and thought that adoptive immunity techniques could answer queries especially on suppressor cells. Thus, cells were transferred from animals variously sensitized with CNS antigens and different adjuvants e.g. alum, IFA or CFA to recipients variously injected with similar preparations. The number of combinations is enormous, however, the two protocols that are most interesting are: 1) Donors sensitized with CNS antigens with IFA and lymph node cells transferred to recipients which are challenged with CNS antigens + CFA, and 2) Donors sensitized with CNS antigens and CFA with lymphocytes transferred to recipients which are sensitized using CNS antigens + IFA. From these experiments, it was clear that suppressor cells were not protective when transferred to an animals actively sensitized for acute or chronic EAE; on the other hand, sensitization with spinal cord and IFA actively protected recipients against adoptive EAE. When both donors and recipients were sensitized using CNA antigens + CFA, recipients were protected. This would ostensibly be due to the type of protection acquired by prior injections of killed mycobacteria. Controls in the form of donors and recipients injected with egg albumin and CFA indicated protection by this means in recipients or stemming from donors. From these results, the potential for increased information by isolation of the suppressive cells from the cytotoxic cells seems great, using rosetting, columns, or cell sorter to acquire active subsets of lymphocytes. We look forward to cooperation with others in the laboratory who are currently using the fluorescence activated cell sorter (FACS II) for determining subsets of T cells in mice.

Formation of Cataracts in Juvenile Guinea Pigs During Acute Stage of EAE

Cataracts were observed in juveniles undergoing the paralysis of acute EAE after transfer of lymph node cells from histocompatible donors. The type of cataracts which were induced in these guinea pigs were later found also in those juveniles acutely paralyzed after active sensitization. Under the slit lamp, corneas, irises and anterior chambers were normal, but lenses showed either a doughnut-shaped or solid opacity. The lens capsule was intact with evidence of posterior subcapsular changes. The lens histology was typical for cataract with no inflammatory cells in vitreous, choroid or retina. To our knowledge, cataracts are not associated with multiple sclerosis for which chronic EAE is a model, the ocular pathology in the human disease being due to central demyelinating lesions or damage to the optic nerve. Spontaneous cataracts in guinea pigs are rare; those we have seen were morphologically different from the experimentally induced ones. The lack of inflammatory cells suggests a non-immunological direct cause, and we have turned to two experimental models based on metabolic changes for hints, the cataracts seen in tryptophane-deficient guinea pigs or rats and in galactosemic rats. Our rationale is based on the association of the cataracts with paralyzed and wasted guinea pigs. Cataracts are observed in humans not able to incorporate glucose into wasting muscle of myotonic dystrophy. Other evidence against the immunologic origin of the cataract is the absence of reports of cross-reactivity between brain and lens antigens.

Future investigation centers around biochemical studies of the altered lens on the one hand, and of the effects of tryptophane and galactose on the model of juvenile EAE on the other hand. In preliminary studies undertaken with our collaborators in the National Eye Institute, there are evidently some changes in the percentages of the different lens crystallines in the cataracts seen in the juvenile guinea pig paralyzed in the acute stage of EAE.

Publications:

Raine, C. S., Traugott, U., Nussenblatt, R. B., and Stone, S. H.: Optic neuritis and chronic relapsing experimental allergic encephalomyelitis: Relationship to clinical course and comparison with multiple sclerosis. Lab. Invest. 42: 327-335, 1980.

Raine, C. S., Traugott, U., and Stone, S. H.: Lymphocyte studies in acute and chronic relapsing EAE during suppression. In, The Suppression of Experimental Allergic Encephalomyelitis and Multiple Sclerosis. Davison, A. N. and Cuzmer, M. L. (Eds.), Academic Press, New York, 1980, pp. 119-140.

Stone, S. H., Nussenblatt, R. B., Cross, F. L., and Raine, C. S.: Cataracts and allergic encephalomyelitis: acute opacification of the lens in paralyzed juvenile guinea pigs. Ophthal. Res., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00203-01 LMI

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Applications of Flow Microfluorometry

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. M. Chused	Senior Investigator	LMI/NIAID
OTHER:	J. D. Longstreth	NIH Postdoctoral Fellow	LMI/NIAID
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	E. Brown	Biologist	LMI/NIAID
	K. J. Kim	Visiting Associate	LMI/NIAID
	C. Kanellopoulos-Langevin	Visiting Associate	LMI/NIAID
	B. E. Seligmann	Research Associate	LCI/NIAID
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	G. Weil	Research Associate	LPD/NIAID
	C. A. Kozak	Staff Fellow	LVD/NIAID
	A. Schultz	Computer Specialist	DCRT/NIH
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COOPERATING UNITS (if any)

None

LAB/BRANCH
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SECTION
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TOTAL MANYEARS: 1.3	PROFESSIONAL: 0.8	OTHER: 0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Flow microfluorometry is being applied to the study of charge sensitive dyes, the purification of human eosinophils, the detection of spontaneous antibody to murine leukemia viruses, somatic cell genetics and the generation of hybridomas.

PHS-6040
(Rev. 10-76)

Project Description:

Flor microbluorometry is a powerful technique in cellular immunology. The recent development of a dual laser fluorescence activated cell sorter (FACS) permits the simultaneous measurement of light scatter (closely related to cell size) and fluorescence in two independent channels at flow rates up to 5000 cells per second. Single parameter analysis, typically using fluorescein conjugated reagents, has already become a fairly standard immunologic technique. The goal of the NIAID FACS installation is to provide as extensive collaborative opportunities as possible to investigators in the Institute as well as to explore new applications of this advanced technology. To this end a number of enhancements have been made in the computer software used with the FACS. Completely new programs have been written in a much more flexible and powerful computer operating system to collect and analyse FACS data. These store a complete description of the material being tested, staining agent(s), etc., and make it available during data retrieval. Exact operating parameters of the instrument are captured automatically allowing the display of fluorescence in terms of molecules of fluorochrome or of conjugated ligand. Each investigator's data is automatically archived for rapid retrieval. Data evaluation and plotting can be set up to proceed automatically for large numbers of files.

To date, twenty-two investigators have used the system. Some of the collaborative projects underway include:

1. The use of charge sensitive cyanine dyes to measure the membrane potential of lymphocytes and their response to various stimuli.
2. The purification of human eosinophils to 98+% based on their spontaneous autofluorescence. Since the cells are unstained they function normally in several assays.
3. The study of spontaneous antibody to various types of murine leukemia viruses by indirect immunofluorescence.
4. The study of murine B cell tumors for evidence of response to B cell mitogens.
5. The isolation and cloning from mouse-hamster somatic cell bybrids of lines expressing different and distinct amounts of murine leukemia virus gp70 in an effort to map the responsible gene(s).
6. The development of a panel of rat anti-NZB spleen hybridomas. An attempt will be made to use the FACS in cloning them.

The only additional wavelength of laser illumination suitable for the FACS is 568 nm. This is only 5 nm below the peak emission of TRITC. For this reason, in collaboration with Marvin Sternfeld of Research Organics, Cleveland, Ohio, a new dye, XRITC, was developed, which emits at 601 nm. This dye serves well in two-color immunofluorescence with the FACS and is currently in use. A number of studies utilizing the two-color immunofluorescence are in progress.

Software has been developed to measure emission anisotropy, a measure of membrane fluidity, which may correlate with lymphocyte activation. In the near future we expect to develop the hardware and software to allow simultaneous immunofluorescence and measurement of fluorescence depolarization in order to begin the study of the relationship of membrane properties with surface differentiation antigens.

Publications:

Chused, T. M., S. O. Sharrow, J. Weinstein, W. J. Ferguson, and M. Sternfeld: XRITC: A new dye for two-color immunofluorescence. J. Histochem. and Cytochem. 1980. (In press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00204-01 LMI

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Immune Responses to Non-H-2 Antigens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	T. M. Chused	Senior Investigator	LMI/NIAID
	B. A. Taylor**		

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NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cytotoxic T lymphocytes (CTL) specific for allelic products of minor histocompatibility locus, H-1, were generated in secondary in vitro cultures. Effector cells required H-2 compatibility between stimulates, responders, and target cells. There was no strict correlation between the strength of CTL responses to minor H antigens and the mean skin graft rejection times for these antigens.

NZB mice respond to non-H-2 antigens after primary stimulation in vitro with H-2 compatible cells. The response is directed against H-2D-associated and Qa-1^{b}-associated as well as other antigens. The response is controlled by two or more genes.

Project Description:

Evaluations of immune responses to non-H-2 antigens are an important aspect of transplantation and tumor biology. We have initiated studies of CTL responses to minor H antigens in hopes of developing a fuller understanding of the cellular interactions reguired for the response to "weak" antigens which might prove useful in generating effective responses to tumor antigens.

CTL responses to H-1. CTL specific for allelic products of H-1 were generated by priming in vivo and secondary stimulation in vitro. The response to H-1 antigens was shown to be H-2 restricted with H-1 being seen in association with either K or D end molecules. Since other minor antigens appear to associate only with H-2D, this result indicates that distinct Ir genes may regulate the responses to different minor H specificities.

NZB mice develop spontaneous autoimmune disease characterized by the development of multiple autoantibodies. Although there is still debate concerning the etiology of NZB disease, several lines of evidence suggest that normal mechanisms of immunologic regulation are disordered in these mice.

First, it has been shown in this Laboratory and a number of others, that NZB B lymphocytes undergo spontaneous polyclonal activation early in life. This is evidenced by a decrease in the ratio of surface IgD/IgM and a marked increase in plasma cell frequency and IgM secretion. Studies with adult thymectomized, irradiated fetal liver reconstituted NZB and NZB nude mice show that NZB B cell dysfunction occurs in the absence of thymus processed cells and may therefore represent a primary B cell defect.

Second, there is evidence that both young and old NZB mice suffer major derangements in the network of T cells that regulates the immune response. In addition, young NZB mice make an unexpected in vitro primary, non-H-2-restricted cytotoxic T lymphocyte (CTL) respose to H-2 compatible cells. In other strains CTL responses to H-2 compatible cells are only detected following prior in vivo priming.

We have attempted to characterise this unusual T cell response further and to use it as a model for NZB T cell dysfunction. A number of questions have been explored: (1) Does the ontogeny of this defect correlate with that of the demonstrated abnormalities of NZB B lymphocytes? (2) Do T cell-specific autoantibodies promote the response? (3) Is the response governed by single or multiple genes? (4) Do the genes which control T anc B cell defects segregate independently? (5) Are conventionally H-2 restricted responses such as those to TNP-modified isologous cells or to H-Y antigens also abnormal? (6) What is the antigenic specificity of the cytotoxic T cells responding to H-2 compatible cells?

The results of this study show that in a semi-quantitative assay system the mean cytotoxic response of NZB cells following stimulation with BALB/c cells was 157 ± 18 X 10^4 cytotoxic units. This value was approximately one third of the mean allogeneic response of 445 ± 33 X 10^4 cytotoxic units obtained when NZB cells were stimulated with C57BL/6 cells. Both responses were abrogated following treatment with hybridoma anti-Thy 1.2 ascites and complement. NZB cells sensitized in vitro with BALB/c cells also caused consistent weak, cross-reactive lysis of $H\text{-}2^b$, $H\text{-}2^k$, $H\text{-}2^q$ and self targets.

The NZB anti-BALB/c CTL response was detectable by two weeks of age and increased to adult levels by six weeks of age. By comparison the NZB CTL response to C57BL/6 cells was detectable by one week of age and reached adult levels of activity between two and four weeks of age. It is notable that hypersecretion of IgM by NZB B cells and plasma cells is also first detectable at two weeks of age.

To determine whether T cell-specific or other autoantibodies contribute to the apparent increase in the frequency of NZB CTL precursors responding to BALB/c cells, NZB mice were depleted of functional B lymphocytes by treatment from birth with anti-IgM antibodies. Anti-IgM-treated mice were monitored for the presence of surface Ig positive (sIg^+) cells and plasma cells by flow microfluorometry using a FACS II. In all experiments μ-suppressed NZB mice gave CTL responses to BALB/c cells which did not differ significantly from those of untreated controls. The NZB CTL response to BALB/c cells is therefore not dependent on the presence of functional B cells or their products for its expression.

In preliminary genetic studies, F_1 mice from crosses of NZB mice with non-autoimmune strains were tested for primary CTL responses to BALB/c cells. (NZB X NZW), (NZB X NFS), (NZB X B10.D2) and (SWR X NZB) F_1 mice all gave mean cytotoxic unit values intermediate between those obtained with NZB cells and those obtained with (BALB/c X NZB)F_1, (BALB/c X B6)F_1 and B10.D2 control populations following stimulation with BALB/c cells. This data suggested that at least one semi-dominant gene was involved in controlling the primary anti-BALB/c CTL response in NZB mice. The genetic influences on the response were explored further in studies with mice derived from crosses of (NZB X B10.D2)F_1 mice with B10.D2. Although they did not form a distinct subset, 32% (19/58) of the backcross mice had CTL responses exceeding the confidence limits for B10.D2 responses, suggesting that the predominant influences on the NZB anti-BALB/c CTL response are exerted by two genes at least one of which is semi-dominant. The (NZB X B10.D2)F_1, (NZB X B10.D2)F_1 X B10.D2 backcross mice and parental mice used in the above study were also tested for abnormalities in B cell surface immunoglobulin phenotype and plasma cell frequency. Using flow microfluorometry and reagents specific for surface IgD and IgM it was shown that a recessive gene or genes controls the level of surface IgD on NZB B lymphocytes and that the plasma cell frequency is controlled by at least two semi-dominant genes. Linear regression analysis of plasma cell frequency and anti-BALB/c CTL activity in individual backcross mice showed that these two parameters are under the control of independently segregating genes. This was further evidenced in studies with NZB X 129 recombinant inbred mice. Of the recombinants studied none showed a primary CTL response to H-2-compatible cells or a phenotypic abnormality in sIgD expression on B cells. By contrast, all NZB X 129 recombinants had significantly more plasma cells than 129 control mice.

A series of experiments was performed to determine whether NZB CTL responses to non-histocompatibility antigens such as TNP-modified isologous cells, differed in either magnitude or specificity from those of other non-autoimmune $\underline{H\text{-}2}^d$ mice. The results showed that TNP-specific NZB CTL do not cause significantly more cross-reactive lysis of TNP-modified or untreated $H\text{-}2^k$, $H\text{-}2^b$ or $H\text{-}2^d$ targets than BALB/c DBA/2 or B10.D2 anti-TNP effector cells. In every experiment, however, the NZB anti-TNP CTL response was slightly higher than that of other $H\text{-}2^d$ strains. It is also notable that spleen cells from NZB female mice primed *in vivo* with spleen cells from NZB male mice mounted an H-Y specific secondary cytotoxic T cell response when restimulated with NZB male cells *in vitro*. This response was unexpected as $H\text{-}2^d$ mice are conventionally low responders to H-Y antigen. Studies are in progress to determine whether NZB mice make abnormal CTL responses to virus-infected isologous cells.

When the unusual primary NZB CTL response to *H-2* compatible cells was first described by Botzenhardt, et. al., it was thought to be directed against minor H antigens. More recently Rich, et. al., have published data which suggests that the effector population is specific for $Qa\text{-}1^b$ antigens. In contrast, Theofilopoulos, et. al., have evidence that the response is directed to murine leukemia viral gp70. Unlike Rich, et. al., we have shown that NZB anti-BALB/c CTL will lyse targets bearing either $Qa\text{-}1^a$ or $Qa\text{-}1^b$ antigens. It is notable however, that the strongest lytic activity was always obtained in $H\text{-}2^d$, $Qa\text{-}1^b$ targets.

In preliminary experiments we have been unable to inhibit the generation of NZB anti-BALB/c CTL by the incorporation of a rabbit antiserum specific for xenotropic viral gp70 in mixed lymphocyte cultures. Studies are in progress to determine whether hybridoma reagents specific for xenotropic, ecotropic and recombinant viral gp70's will inhibit the response.

In summary, the results of this study show that the unexpected primary *in vitro* CTL response of NZB spleen cells to *H-2* compatible cells (1) can be induced as early as two weeks of age; (2) is not dependent on the presence of functional B cells or their products, and (3) is controlled by at least two semi-dominant genes which are different from the genes controlling abnormalities in plasma cell frequency and surface IgD expression of B lymphocytes. NZB mice also showed a slightly increased CTL response to TNP-modified isologous cells and an unexpected secondary CTL response to H-Y antigen. The nature of the antigen(s) recognized by NZB T cells on BALB/c or other $H\text{-}2^d$ cells remains to be resolved. Our data would suggest that the response may be multispecific with both minor H antigens and $Qa\text{-}1^b$ antigens being recognized. T cell cloning studies which are in progress may help to resolve this issue.

In conclusion, the data presented here together with the lack of evidence for abnormalities in suppressor T cells in young NZB mice are consistent with the view that NZB CTL precursors specific for antigens coded for outside of H-2 have undergone early and abnormal clonal expansion in vivo.

Publications:

Dunlop, M.B.C., Morse, H.C. III, and Graff, R.J.: The cytotoxic T-cell response to H-1 minor histocompatibility antigen differences. Cell. Immunol., 50: 169-176, 1980.

Laboratory of Parasitic Diseases
1980 Annual Report
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Laboratory of Parasitic Diseases

National Institute of Allergy and Infectious Diseases

SUMMARY - October 1, 1979 through September 30, 1980

INTRODUCTION

The Laboratory of Parasitic Diseases was pleased to have Russell Howard, Ph.D. of Australia join the Malaria Section as an Expert Consultant. New Guest Investigators under IPA auspices include Robert Genta, M.D., Rabia Hussain, Ph.D., and Edna Kaneshiro, Ph.D. Although only at the LPD for a few months, Eloi de Souza Garcia, Ph.D., kept many of us hopping while here as a Guest Investigator. The new Staff Fellow is John Barnwell, Ph.D. M.K. Wassef, Ph.D. came as a Senior Staff Fellow but accepted a position for the Center for Disease Control, so he now retains a Guest Worker assignment. There are four new Visiting Fellows: Iroka Udeniya, Ph.D. of Nigeria, who just finished his doctorate at West Virginia. Aldine Barral, M.D. of Brazil and two from England, Patricia Graves, Ph.D. and Peter Gardiner, Ph.D. Only one new Research Associate, Terence Hadley, M.D. joined us this year. An additional tenured investigator, Dr. Sher is expected in the fall, along with several research fellows. Gabriel Schmunis, M.D., an Expert Consultant from Brazil, left to take a position with the Pan American Health Organization, and Dr. Charles Richards retired from the U.S. Public Health Service but retains an association with the LPD as a contractee for work on snails. John Sullivan, Ph.D., a Guest Investigator with Dr. Richards, left to take a position with the University of South Carolina. Finally, we were pleased to have Dr. Leon Jacobs, a former Laboratory Chief of the LPD, come back with us as Emeritus Scientist.

Field research in India continued with Drs. Ottesen and Weil spending several weeks in Madras on the filariasis studies. Dr. Hofstetter and Mr. Poindexter worked together with our CDC collaborators on schistosomiasis in Puerto Rico. As a result of Dr. Neva's short trip to the Dominican Republic, four leishmaniasis patients came to the Clinical Center for study, and then Dr. Petersen of LCI and Dr. A. Barral of LPD followed this up later with a visit to the Dominican Republic for further field work. Late in the fiscal year Dr. Ottesen returned to India again to complete special work on filariasis.

Laboratory of Parasitic Diseases

National Institute of Allergy and Infectious Diseases

SUMMARY - October 1, 1979 through September 30, 1980

RESEARCH ACCOMPLISHMENTS

HELMINTHIC INFECTIONS Allergy, IgE and the Eosinophil: Allergic, or immediate hypersensitivity, reactions are not very common in chronic parasitic infections. Evidence has now been found for "blocking antibodies" in the serum of patients with chronic filariasis which inhibit antigen-specific histamine release, the classic indicator of allergic reactions. (Ottesen). This helps explain why hives and other allergic signs are not prominent in those patients in whom the parasite (antigen) persists for long periods in the circulation. A radio-sorbant (RAST) type antibody test for parasite-specific IgE has been developed in which antigen is coated on sepharose beads and reacted with the test serum,and then exposed to radio-labelled anti-human IgE. As little as one ng/ml of serum IgE can be detected and anti-parasite IgG was shown not to affect the test. (Hussain and Ottesen). The issue of pre-natal immune response to parasite antigens was studied by testing 40 pairs of maternal and cord bloods from India. Eight of the mothers had microfilaremia and one-fourth of all cord sera had elevated levels of IgE. Since IgE does not cross the placenta, this suggests that some type of pre-natal sensitization to filarial antigen can occur. (Weil, Ottesen and Indian collaborators). The role of the eosinophil in allergic and certain parasitic infections has been of great interest, so better methods for separating these cells from other element of the blood would be useful. By taking advantage of the autofluorescence of eosinophil granules, a separation technique was devised using the fluorescence-activated cell sorter. Eosinophils recovered by this method were viable and functional *in vitro*. (Weil and Chused of LMI).

Improvements in Serodiagnosis: Polyacrylamide gel electrophoresis combined with radioautography and radio-immunoelectrophoresis to identify relevant antigenic components are being used to characterize stage-specific parasite antigens. In addition, solid phase radioimmunoassays have been developed to permit measurement of parasite specific immunoglobulin levels. With such an assay for filarial antigen, as little as one ng of IgE protein/ml of serum can be detected, and specific antifilarial IgE levels in an endemic population ranged from 4 to 600 ng per ml. (Hussain and Ottesen). Work is in progress to develop similar tests for schistosome and strongyloides stage specific antigens, including those associated with excretory-secretory products of the parasites. (Hussain, Nuzhat, Nash and others). The schistosome polysaccharide antigen-antibody systems previously described which provide evidence of either duration or intensity of infection have been refined by using radio-immuno instead of ELISA assays. (Nash). The periodic availability of human strongyloides larvae from a heavily infected patient with an immune defect stimulated our interest in better antibody tests for this infection. Using cultured larve from human and later with rat

Strongyloides parasites, promising results have been obtained in an ELISA-type serologic test for infection with this parasite. (Neva and Gam).

Pathogenesis of Chronic Helminthic Infections: While host immune response and changes in this response with time are important considerations, it is necessary to assess the actual pathologic consequences of chronic helminthiases. In this case comparative studies of a parasite in different hosts, or related parasites in the same host can be useful. Different in-bred strains of mice were found to show marked variation in collagen levels and fibrosis around schistosome egg granulomas. These findings could not be related to H-2 haplotypes. Striking reversal of severe hepatic fibrosis was found after treatment of rabbits infected with *S. japonicum.* In spite of the excellent model which the rabbit provides for hepatic fibrosis due to *S. japonicum*, this host gave anomalous results as an indicator of worm burden with a Japanese as compared to a Phillipine strain of parasite. (Cheever). Thus, the results in a single host species may not adequately characterize a schistosome strain parasitologically or pathologically. An adequate but variable blastogenic response of peripheral blood mononuclear cells to schistosome antigens was found in monkeys infected with *S. japonicum.* (Cheever, Barral and Ottesen). However, a pattern of blastogenic responses to schistosome antigens similar to patients with *S. mansoni* was found in a group of Laotian refugees infected with the newly described schistosome from S.E. Asia, *S. mekongi.* This finding is of considerable interest since this is virtually the first information on cell-mediated immune responses in oriental schistosomiasis, and also because there is accumulating information that some aspects of immune response in *S. japonicum* infections are unique. (Cheever, Ottesen, Barral and Hofstetter).

CELL BIOLOGY AND PHYSIOLOGY OF SOME INTRACELLULAR PROTOZOA

Leishmania: The systematic study of leishmanial surface membranes has disclosed the basis for antigenic sharing between insect and culture form parasites as well as different species of hemoflagellates. At least one common triton-extractable, surface membrane component was found in *L. donovani* amastigotes and promastigotes. Antibodies against this component were found in sera of patients with Kala azar and cutaneous leishmaniasis. (Dwyer). A polysaccharide from surface membranes of 3 species of leishmania, as well as *T. cruzi* was found to be antigenically identical. (Dwyer and Gottlieb). The presence of an acid phosphatase and 5' and 3' nucleotidase enzymes, on the outer lamina of surface membrane of *L. donovani* has been localized and characterized enzymatically. The significance of these enzymes is not yet known but is likely to be unusual since the acid phosphatase is usually considered a lysosomal enzyme and the 3' nucleotidase is unique to animal cells. (Dwyer and Gottlieb). Colloidal gold was found to be a useful lysosomal label for study of leishmania by electron microscopy - it was even taken up by macrophages *in vivo* after injection of infected animals. (Berman and Dwyer). Cultures of human monocyte-derived macrophages continued to be useful for evaluating effects on intracellular forms of leishmania. It was possible to demonstrate antigens of *L. tropica* and *L. donovani* on surface membranes of such cells. The system was also found to be useful for testing the action of drugs and the effect of heat upon intracellular forms of leishmania. (Berman and Dwyer). Additional strains of leishmania from patients with cutaneous disease have been tested in footpads of BALB/c mice to indicate that spectrum of responses can result varying from nothing to striking lesions that develop within 3 to 5 weeks. The concentration of viable organisms within biopsied skin lesions of patients was found to be as high as 10^4 or 10^5 organisms per mg of tissue. (Neva).

Trypanosoma cruzi: Many lines of evidence are leading to a genetic approach with this parasite that causes Chagas' disease; i.e., that within a population of organisms there may be subpopulations with various attributes, including ability to cause disease. Clones of epimastigotes were derived by single cell isolation from several different isolates and found to retain stable isoenzyme patterns after serial passage for 6 months. Surprisingly, doubling times of the clones varied from about 40 hours to as long as about 185 hours. It appears also that these clonal growth rates for epimastigotes hold for the clone in vertebrate cell cultures. (Dvorak and Miles of Brazil). Two of the epimastigote clones of *T. cruzi* were found to have strikingly different characteristics in their growth after being fed to vector bugs. One clone transformed readily to metacyclic trypanosomes in the bug, but the other clone failed to undergo metacyclic transformation. The epidemiologic implications of this finding, if extended, could be very important since the metacyclics are infective stages of the parasite. (Dvorak and Garcia). A different approach to recognition and selection of clones of intracellular forms of *T. cruzi*,that has been described before, is the formation of plaques on a monolayer of cells under agar. (Neva). By using human fibroblasts (Lesch-Nyhan) deficient in the enzyme needed to incorporate guanine into RNA of the cell, it was possible to show that synthesis of RNA by intracellular *T. cruzi* was not required until after reorganization of the parasites had occurred. (Crane and Dvork). Further information is now available concerning the lytic antitrypanosome factor (ATF) produced by growing cells of several species of *Pseudomonas* bacteria. The active susbstance is a small molecule (6,000 to 12,000 daltons) and is active against other trypanosomes and leishmanial flagellates as well. (Mercado).

BIOCHEMISTRY AND PHYSIOLOGY OF LUMINAL PROTOZOA

Giardia lamblia: The ability to grow *Giardia* in culture is providing the opportunity to learn more about this parasite. A new human isolate was established in culture with the addition of fresh liver to cultures (Gillin) and bile supplementation was found helpful for other established strains. (Keister). Further evidence was found for presence of thiol groups on the surface of giardia trophozoites. *In vitro* sensitivity of the parasite to drugs gave results as would be expected from clinical experience with patients. (Gillin). The iron-sulfur electron transfer protein of *Giardia* is clearly different from that of *E. histolytica*, exhibiting its characteristic EPR signal in the oxidized state, perhaps reflecting its different G-I habitat as contrasted to amebae. (Weinbach, Claggett, Keister). With antigens prepared from cultured *Giardia*, antibodies in the IgG class could be measured in patients by the ELISA test and it should be possible to develop assays for antibodies in all Ig classes. Transformation of lymphocytes after exposure to similar antigens has been demonstrated in infected patients. (Smith). An assay for direct immune cytotoxicity against the parasite has also been developed. (Smith and Keister), so it is now possible to examine a wide range of immunological response to this infection. Infectivity of human *Giardia* for the newborn mouse has been confirmed (Keister and Mattern), providing an animal model that may have advantages for certain experimental studies.

Entamoeba histolytica: A mixture of defined components was found to serve adequately as a replacement for yeast extract, one of three undefined components of medium for axenic cultivation of *E. histolytica*. But, adequate replacements for the other two components, trypticase and serum, still have not been found. (Diamond). Studies with a succinylated derivative of Con A (S-Con A) indicate that surface antigens of amebae that bind Con A are involved in attachment of trophozoites to surfaces. Inhibition of amebic attachment by S-Con A was not due to agglutination or capping and was not produced by exposure to anti-amebic sera or to other lectins. (Gillin). The toxin produced by *E. histolytica* that produces detachment of cells from surfaces was found to be neutralized by IgG in the serum of patients with invasive amebiasis. (Mattern). A hemagglutinating lectin derived from amebae and described by others was found to share certain properties with the amebal toxin, but to also have significant differences. (Mattern and Israeli collaborators). An interesting example of the critical importance of composition of medium upon biochemical characteristics of amebae was found. Axenically cultivated amebae grown in a new "improved" TYI-33 medium failed to oxidize L-serine, whereas the same strain of parasite grown and frozen in old TP-S-1 medium was able to metabolize this amino acid. (Weinbach, et al). The finding of reduced levels of serum iron and iron binding capacity in Mexican patients with amebic liver abscess is similar to that found in other acute microbial infections, but the significance of these changes remains obscure. (Diamond and Mexican collaborators).

Tritrichomonas muris: Some characteristics of this intestinal protozoan of hamsters may provide some useful clues to transmission of the human trichomonad, *T. vaginalis*. Pseudocysts, without a true cyst wall, were found to be excreted in the feces and to "excyst" into motile trophozoites. These pseudocysts are likely responsible for infection of new born hamsters. (Mattern and Daniel).

ARTHROPOD AND SNAIL VECTORS OF PARASITES

Transformation of T. cruzi in the bug: A highly specific and active enzyme, pepstatin, which inhibits breakdown of the blood meal hemoglobin in the gut of the triatomine vector bug also prevents molting and other physiologic events in the bug. Since the parasite, *T. cruzi*, transforms from epimastigotes to infective metacyclic trypanosomes in the bug, the effect of ingestion of pepstatin with the blood meal was tested upon subsequent transformation of *T. cruzi*. The fact that pepstatin had no influence on transformation of the parasite indicates that the parasite utilizes directly from the blood meal whatever is necessary for transformation, without some initial processing by the vector bug. (Garcia).

Snail vectors of schistosomes. Even after deliberate attempts there was no indication of induced resistance to the 4 mulluscicides Frescon, pentachlorophenate, Bayluscide and copper sulfate, in two different stocks of the vector snail for schistosomiasis, B. glabrata. Thus, it would appear that if vector resistance develops during intensive use of molluscicides in control programs it would be due to mutation rather than selection. B. glabrata, when infected with x-irradiated miracidia of certain species of trematodes, acquires specific resistance to subsequent challenge with non-irradiated miracidia of the same species. (Sullivan).

Anopheline mosquito physiology: The hypothesis that antibody directed against gut secretions or hormones vital to the insect's normal physiology could adversely affect a hematophagous insect vector is supported in the case of triatomine bugs but not, thus far, in mosquitoes. Delays in molting and reduced egg production was observed in R. prolixus fed upon guinea pigs immunized with secreting guts of the same species of bug, but no effect was seen in mosquitoes fed on mosquito gut antiserum. (Rosenberg). The inability of malaria oocysts to develop normally in certain anopheline mosquitoes, while developing normally to infective sporozoites in other species has been recognized previously. However, a new locus in this barrier to sporogonic development has been discovered in which P. knowlesi develops oocysts and even releases apparently normal sporozoites in An. freeborni, but the salivary glands fail to become infected. (Rosenberg). Additional evidence for the existence of a complex of subspecies within Anopheles balabacensis, an important Southeast Asian malaria vector, is the finding of isoenzyme differences in two populations of this mosquito present in the laboratory. In addition, a factor which promotes synchronous hatching of eggs has been found to be associated with water containing larvae of the homologous population. (Rosenberg).

MALARIA RESEARCH

Hybridoma technique to produce monoclonal antibodies: The hybridoma technique, fusing spleen cells from immunized mice with cells from a myeloma line, and then selecting hybrid cells which produce a single antibody to a desired antigen, has produced several monoclonal lines to P. gallinaceum gametes. Each antibody alone reacts with gametes, but only when combined together do two of the antibodies react sufficiently with male gametes to reduce their infectivity for mosquitoes. One of these antibodies reacts with a gamete protein of apparent molecular weight of 225,000 daltons by immunoprecipitation. (Rener, Carter, Miller and Howard). With P. knowlesi, three monoclonal antibody lines have been found which agglutinate merozoites and bind to the merozoite surface. However, only one of these antibodies shows some functional effect on invasion and the effect is not striking. There are some discrepancies on the basis of molecular weight in the antigens that can be identified from schizont preparations as compared to merozoites. (Epstein, Miller, Udeniya and Kaushal).

Gamete physiology and immunology: Even though several important factors governing gametocyte production in vitro have been identified, we have still been frustrated in obtaining regularly and reproducibly gametocytes that are infective for mosquitoes. A plateau phase in which maximum asexual growth has already been attained is one stimulus, and addition of cylic AMP to cultures at an appropriate time in the growth cycle has a dramatic effect upon conversion to gametocytes. However, the effect of cyclic AMP produces almost entirely gametocytes of one sex with the isolate of *P. falciparum* being cultured, and the propensity to form gametocytes of whichever sex differs greatly from one line of parasite to another. (Kaushal, Carter and Miller). In the *in vivo* situation, optimum infectivity of gametocytes is reduced with both avian and some simian malarias when asexual parasitemia is maximum. This reduced infectivity cannot be ascribed to antibodies which block transmission. Yet, factors associated with erythrocytes seem necessary for maximum infectivity of gametocytes for mosquitoes. (Rosenberg). Rhesus monkeys immunized over 3 years ago with *P. knowlesi* gametes in Freund's complete adjuvant continued to show high levels of anti-gamete antibodies and, when infected, blocked transmission effectively in mosquitoes. (Gwadz).

Malarial merozoites and merozoite invasion of red cells: Considerable emphasis is being put upon the merozoite, its antigens and antibodies that react with it since it is the infective stage for the asexual cycle of multiplication. ^{125}I-lactoperoxidase labelled merozoites give a characteristic profile on acrylamide gel electrophoresis. Trypsin-treated merozoites that can no longer attach or invade red cells lose the high molecular weight proteins (>100,000 daltons) with the appearance of new bands at 60,000 to 70,000 daltons. Thus, the high molecular weight proteins are candidates for the receptor material. Con A agglutinates *P. knowlesi* merozoites and fluorescin-labelled Con A is seen on the surface of merozoites. It was then found that parasite proteins metabolically labelled with ^{35}S-methionine were bound to Con A columns and could be specifically eluted with α-methyl mannoside. It is hoped that this method may be used to partially purify membrane glycoproteins. It was reported last year that antibodies to *P. falciparum* cross-reacted with surface determinates on *P. knowlesi* merozoites. Such antibodies were also found in the serum of Gambians who were clinically immune to *P. falciparum*, but these sera did not block invasion of red cells by *P. knowlesi* merozoites. (Johnson, Epstein, Carter, Miller and Howard).

Mosquito vectors for sporozoites and for drug screening: A collaborative project is continuing which supplies irradiated sporozoites of *P. knowlesi* for immunization of monkeys. Sporozoites immunity was found to be strain specific for two geographic varieties of *P. knowlesi*, one from the Phillipines and the other from Malaysia. Monoclonal antibodies from hybridomas are also being produced against *P. knowlesi* sporozoites. (Gwadz in collaboration with Nussenzweig of NYU). A procedure was developed for screening for anti-malarial drugs with activity against hepatic schizonts. Drugs with known activity against the exo-erythrocytic forms, also appear to have gametocytocidal activity in the *P. gallinaceum*/chicken/*A. aegypti* system. (Gwadz, Koontz and Miller).

Laboratory of Parasitic Diseases

National Institute of Allergy and Infectious Diseases

SUMMARY - October 1, 1979 through September 30, 1980

HONORS AND AWARDS

Dr. Richard Carter was an invited participant in a special parasitology course at Woods Hole, Massachusetts sponsored by the Rockefeller and McConnell Clark Foundations.

Dr. Allen W. Cheever serves on the Editorial Board of the American Society of Tropical Medicine and Hygiene.

Dr. Diamond is on the Editorial Board of Experimental Parasitology, was re-appointed to the WHO Expert Advisory Panel on Parasitic Diseases, and was a Consultant and Visiting Scientist at the Weizmann Institute of Israel for 6 weeks.

Dr. Dvorak served as coordinator to the National Science Foundation's proposed trilateral collaborative research program, gave an invited lecture for the President's Symposium at the annual meeting of the American Society of Parasitology Meeting, and was co-chairman of a symposium at the 2nd International Congress on Cell Biology in Berlin.

Dr. Dwyer serves on the Editorial Board of the Journal of Protozoology, and was awarded the Henry Baldwin Ward Medal of the American Society of Parasitologists, an award given to the Society's most outstanding young research worker.

Dr. Teresa Mercado accepted an appointment by Dr. Frederickson to an 8-member Task Force to evaluate the Division of Equal Opportunity of the NIH and served as a panelist of a workshop on Opportunities for Minority Students in Graduate Education, Arts, Sciences and Engineering.

Dr. Louis H. Miller serves on Editorial Boards of Experimental Parasitology, J. of Parasitology and J. of Molecular Medicine. He continues to serve as member of the Steering Committee for Immunology of Malaria of the TDR of WHO.

Dr. Theodore Nash serves on the NIAID Clinical Research Committee and was elected to membership in the Infectious Disease Society of America.

Dr. F.A. Neva serves on the Editorial Board of the American Journal of Tropical Medicine and Hygiene and completed service on the NAS-Institute of Medicine's study on Clinical Investigation in Developing Countries.

Dr. Eric Ottesen was co-author of two different papers awarded special prizes (Clinical prize of ASTM&H in 1979 and Berson-Yalow award of Society of Nuclear Medicine 1980); is on Editorial Board of Experimental Parasitology and was appointed to the Steering Committee of the Scientific Working Group on Filariasis of the TDR of WHO.

Dr. Eugene Weinbach was appointed to the Editorial Board of the Journal of Parasitology.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00091-18 LPD

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Genetics, Biology, and Control of Snail Intermediate Hosts of Schistosomes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. T. Sullivan Guest Worker, LPD, NIAID

COOPERATING UNITS (if any) Dr. K. J. Lie and Dr. Donald Heyneman, The George Williams Hooper Foundation, University of CA., Dr. C. S. Richards, Biomedical Research Institute, Bethesda, MD.

LAB/BRANCH

Laboratory of Parasitic Diseases

SECTION

Host-Parasite Relations Section

INSTITUTE AND LOCATION

NIAID, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	-

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

B. glabrata, when infected with x-irradiated miracidia of certain species of trematodes, acquires specific resistance to a subsequent challenge with non-irradiated miracidia of the same species. In addition, preinfection with nonschistosome miracidia interfers with the active resistance of the snails to S. mansoni, rendering them temporarily susceptible. These phenomena are being studied with the trematodes Echinostoma paraensei and Ribeiroia marini.

The ability of three stocks of B. glabrata to develop resistance to the 4 most commonly used molluscicides is being compared. Successive generations of survivors of molluscicide exposures are being reared and tested for molluscicide resistance.

PHS-6040
(Rev. 10-76)

Project Description:

This project continues many aspects of the work of the former PI, Dr. C. S. Richards, who retired at the end of the previous fiscal year.

Mortality regression lines have been calculated for PR-79 and M-line stocks of Biomphalaria glabrata exposed to four molluscicides: Frescon, pentachlorophenate, Bayluscide, and copper sulfate. Based on results of successive exposures of offspring from LC_{90} survivors for 2-3 generations there appears little indication of development of resistance to any of the 4 molluscicides in either stock of snails. The two parental stocks are equally susceptible to Frescon and pentachlorophenate. However, the LC_{90} of M-line is several-fold greater for both Bayluscide and $CuSO_4$ than that of PR-79, despite collection of the latter snails from an area previously exposed to molluscicides. Attempts to cross-breed the two stock of snails in order to assess the susceptibility of the F_1 hybrids have been unsuccessful. However, separate exposure of pre-cross progeny by selfing from 10 snails of each stock has shown differential susceptibility to Bayluscide between the two stocks and between individuals within each stock.

LC_{50} values have been obtained for the one double bond (1.40 ppm) and three double bond (0.36 ppm) forms of cashew nut shell anacardic acid, and that of the two double bond form appears to be intermediate, approximately 1.0 ppm.

The study of acquired resistance to Ribeiroia marini has been completed. This resistance is quite similar in most respects to that reported against echinostomes, except that snails sensitized to R. marini also acquire nonsusceptibility to heterologous species. In order to study the role of the amebocyte producing organ in B. glabrata, snails have been irradiated before sensitization. Although irradiated snails are still capable of acquiring resistance to R. marini, susceptibility of irradiated snails, whether sensitized or nonsensitized, to normal R. marini is significantly greater than that of the corresponding nonirradiated snails; i.e., irradiation increases snail susceptibility.

Studies on nonsusceptibility of B. glabrata to PR-2 S. mansoni are continuing. By employing a descriptive ranking system based on the condition of sporocysts found in tissue sections of snails and their relative prevalence, a range of nonsusceptibility has been discovered in 5 genetic stocks of snails, from stocks that destroy all sporocysts within 3 days to others in which sporocysts persist undeveloped yet viable for 3 weeks. An analogous range of susceptibility is being observed in 3 stocks of susceptible snails. Preliminary results of exposures to PR-2 of snails infected 5-6 days previously with nonirradiated E. paraensei show that most stocks of PR-2 nonsusceptible snails revert to partial susceptibility when infected with the echinostome. Therefore, nonsusceptibility among these stocks is most likely to immunologic resistance, rather than biochemical unsuitability. Without exception, all F_1 hybrids from crosses involving a nonsusceptible parent are themselves nonsusceptible to PR-2. Interestingly, all nonsusceptible stocks thus far crossed with 10-R2 have yielded F_1 offspring that display the characteristic tissue reaction of the 10-R2 parent, i.e., destruction of all sporocysts within 3 days. Histological examination of PR-2 in F_2 and F_3 offspring resulting from crosses among the various stocks is now underway.

This project has been discontinued following the resignation of Dr. Sullivan.

Publications:

1. Kassim, O. O. and Richards, C. S.: Radioisotope labelling of Schistosoma mansoni miracidia for in vivo studies in Biomphalaria glabrata. J. Invert. Pathol., 33: 385-386, 1979.

2. Kassim, O. O. and Richards, C. S.: Host reactions in Biomphalaria glabrata to Schistosoma mansoni miracidia, involving variations in parasite strains, numbers and sequence of exposures. Internat. Jour. for Parasit. 9: 565-570, 1979.

3. Lie, K. J., Heyneman, D., and Richards, C. S.: Specificity of natural resistance to trematode infections in Biomphalaria glabrata. Internat. Jour. for Parasit., 9: 529-531, 1979.

4. Kassim, O. O., Cheever, A. W., and Richards, C. S.: Schistosoma mansoni: Comparison of infections in mice with different strains of worms. Exp. Parasit., 48: 220-224, 1979.

5. Poinar, G. O., Jr. and Richards, C. S.: Daubaylia helicophilus n. sp. (Daubayliidae: Nematoda) a parasite of the snail Gyraulus spirillus from Taiwan. Annales de Parasitologie (Paris), 54: 615-619, 1979.

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9. Richards, C. S.: Abnormal growths from the digestive gland of Biomphalaria glabrata. J. Invert. Pathol., 35: 318-319, 1980.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00092-14 LPD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
The pathogenesis of schistosome infections in mammalian hosts.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. W. Cheever	Assistant Chief, LPD, NIAID
Other:	T. E. Nash	Medical Officer, LPD, NIAID
	E. A. Ottesen	Medical Officer, LPD, NIAID
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	M. Barral	Kellogg Research Fellow, LPD, NIAID

COOPERATING UNITS (if any) Southwest Foundation for Research & Education, San Antonio, TX (Dr. Kuntz); Dept. Gastroenterol., WRAIR (Dr. Dunn); Lab. Statistical and Math. Methodology, DCRT, NIH (Dr. Mosimann, Mrs. Minker); Div. Immunoparasitology, NMRI (Dr. Dean), American Foundation for Biological Research (Dr. Richards).

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Host Parasite Relations

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	2.0	1.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Schistosome infections are studied quantitatively in man and experimental animals. Present studies emphasize fibrosis induced by schistosomiasis and resistance to reinfection with S. mansoni in laboratory mice. Initial studies show the fibrotic response to eggs in the liver of S. japonicum infected rabbits is much more marked than in the intestine. Mouse strains show marked differences in hepatic fibrosis. This has not yet been shown to have a genetic basis.

The nature and pathogenesis of lesions produced in mammalian hosts by schistosomes of medical importance are studied in man and experimental animals. Schistosome infections are quantitated by measurement of worm numbers, passage of eggs in excreta and accumulation of eggs in host tissues.

Hepatic fibrosis. Fibrosis of the granulomas around *Schistosoma mansoni* eggs in the liver shows marked variation in different species and in different strains of mice. C57BL mice consistently showed the least fibrosis while several outbred strains and the NMRI inbred strain had maximal levels of collagen. Collagen levels were not related to H-2 hapoltypes or to the size of the granulomas formed around the eggs. In vitro rates of collagen synthesis paralleled the liver collagen content. We are presently screening a number of inbred strains hoping to find a well characterized "high responder" which will facilitate study of F-1 and backcross generations.

The reversal of severe hepatic fibrosis is being studied in *S. japonicum* infected rabbits which are given schistosomicidal drugs 20 weeks after infection. The hepatic fibrosis is remarkably reversible, and by 40 weeks after treatment the collagen content of the liver is near normal. Symmers' fibrosis and cirrhsis gradually disappear during this interval. The fibrotic response to eggs in the gut is much less than that in the liver, confirming results with *S. mansoni* in the liver and gut of the mouse.

Resistance to reinfection with S. mansoni. Recently published experiments suggest that the some laboratory stocks of *S. mansoni* show antigenic diversity which markedly affects resistance to reinfection. We have begun experiments in collaboration with Dr. Charles Richards which utilize the highly selected and inbred strains of *S. mansoni* which he has developed during the past several years. We hope to find or develop stable substrains of worms which differ markedly in antigens relevant to the induction of resistance. We have no results to report as yet but expect that these experiments will occupy a major part of our time in the next two years.

S. japonicum infection in monkeys. Rabbits infected with a Philippine strain of *S. japonicum* execreted very few eggs in the feces (84 ± 21 per day per worm pair) compared to 289 ± 41 in rabbits infected with a Japanese strain of the worm. In rabbits infected with the Philippine strain the number of eggs in the feces was also poorly correlated with the number of worms present. We repeated these studies in monkeys because of the practical importance of evaluating the intensity of schistosome infection in man by the counting of eggs in the feces. Monkeys (*Cebus apella*) infected with either the Philippine or the Japanese strain passed more than 1,000 eggs per worm pair per day in the feces, and passage of eggs in the feces accurately reflected the intensity of infection in the monkeys. The sequestration of schistosome eggs in the tissues of rabbits infected with the Philippine strain thus appears to be an isolated finding which is probably unimportant for the evaluation of infected persons. Other parasitological parameters, such as the distribution of schistosome eggs in the monkey tissues, were characteristic for each worm strain. However, the distribution of lesions and eggs of the two strains in monkeys bore no parallels with their distribution in rabbits. Thus a single host species is not adequate to characterize a schistosome strain parasitologically or pathologically. Preliminary evidence suggests that the Japanese strain has changed considerably in its behavior over the seven years we have used it.

In vitro blastogenic responses of peripheral blood mononuclear cells of S. japonicum infected capuchin monkeys were highly variable but at least as marked as the comparable responses of S. mansoni infected animals. The heterogeneity of response was not correlated with intensity of infection and was only partly explained by suppressive factors in the serum. Studies of antibody response, immune complex and lymphokines in these monkeys continue.

Understanding of the lesions caused by schistosomiasis in man, the associated morbidity and their relation to intensity of infection are basic to evaluating the importance of the disease and to some aspects of control and treatment. The detailed study of animal models contributes to our understanding of the parasite and its possible interactions with the human host.

Publications:

1. Kassim, O. O., Cheever, A. W. and Richards, C. S.: Schistosoma mansoni: comparison of infections in mice with different strains of worms. Exp. Parasit., 48: 220-224, 1979.

2. Cheever, A. W., Duvall, R. H. and Minker, R. G.: Quantitative parasitologic findings in rabbits infected with Japanese and Philippine strains of Schistosoma japonicum. Am. J. Trop. Med. Hyg. (in press).

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4. Cheever, A. W., Duvall, R. H. and Minker, R. G.: Extrahepatic pathology in rabbits infected with Japanese and Philippine strains of Schistosoma japonicum and the relation of intestinal lesions to passage of eggs in the feces. Am. J. Trop. Med. Hyg. (in press).

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00093-05 LPD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Studies of the Immunologic Response to Helminth Infections

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	M. Hofstetter	Research Associate, LPD, NIAID
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	M. Barral	Visiting Fellow, LPD, NIAID
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	T. E. Nash	Senior Investigator, LPD, NIAID
	A. W. Cheever	Assistant Chief, LPD, NIAID
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COOPERATING UNITS (if any) Tuberculosis Chemotherapy Center, Madras, India (Dr. S.P. Tripathy); Medical College of Madras, India (Prof. K.V. Thiruvengadam; San Juan Laboratory, CDC (Mr. E. Ruiz-Tiben); Bureau of Veterinary Research, FDA, Beltsville, Md. (Dr. K.G. Powers).

LAB/BRANCH
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INSTITUTE AND LOCATION
NIAID, Bethesda, Md. 20205

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☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major goals of this project are to characterize the host's immune response to helminth infections and to relate the findings to the pathogenesis of clinical disease. In filariasis the heterogeneity of disease expression may relate to variable patterns of immune recognition and response or to pre-natal exposure to parasite antigen. Immunochemical analysis of serum from patients & neonates to detect the relevant immunogens and allergens of the parasite are under way. The role of immune complexes in the pathogenesis of filariasis and schistosomiasis is also being explored.
Immunologic hyporesponsiveness to parasite antigens is characteristic of chronic helminth infections. Humoral and cellular suppressive mechanisms which regulate the lymphocyte responsiveness in human schistosomiasis are being defined as are changes in responsiveness following treatment of the infection. Immediate hypersensitivity (IgE) responses have been found to be modulated by IgG blocking antibodies which develop in human filariasis and schistosomiasis. Eosinophils are prominent in the host response to helminth infections. Purification of these cells has been achieved with the flourescence activated cell sorter.

Project Description:

The character and evolution of the immune response in man and experimental animals has been studied during and following infection with parasitic helminths.

Filariasis. The questions of why some individuals in an endemic area become infected with filaria (*W. bancrofti*) while others appear to resist it and of why even among infected individuals the clinical manifestations of disease can be so varied have led to a search for the relevant immunologic parameters both in field studies of human filariasis and in work on laboratory models of the disease. Field studies have been carried out in the Cook Islands (Project #J0233-I-74) and in Madras, India (Project #76-I-231), this latter project continuing as an on-going collaboration.

At one end of the clinical spectrum of human filariasis is tropical eosinophilia (TE), a syndrome characterized by chronic lung disease with asthma, profound eosinophilia, markedly elevated IgE levels and high filarial antibody titers. Using an *in vitro* correlate of IgE-mediated allergic responses (*i.e.*, histamine release by IgE-coated basophils challenged *in vitro* with the sensitizing allergen) and radioallergosorbent techniques (RAST) for the detection of specific IgE antibodies, we have documented and quantified the high degree of allergic sensitization to filariae in patients with TE (compared to those with other forms of filarial disease). The especial hypersensitization to microfilariae and their products may be most important in determining the symptoms and pathology associated with this syndrome (Ottesen, Neva, Hussain, Anwar). In other patients whose disease is manifested by elephantiasis and recurrent episodes of lymphangitis (filarial fevers) the finding of high levels of circulating immune complexes suggests a role for these complexes in the immune injury found in such patients. Definition of the determinants and make-up of these complexes is underway (Ottesen, Lawley).

The possibility that pre-natal conditioning of the immune system, by altering the host's response to subsequent infection, may contribute to the heterogeneity of disease expression has been explored in studies of 40 pairs of maternal and cord blood collected in Madras. One fifth of the mothers (but no babies) were microfilaremic. One fourth of all cord sera had elevated levels of IgE (an immunoglobulin which does not cross the placenta), and many contained filaria-specific IgE antibody (determined by RAST). These findings suggest that pre-natal sensitization to filaria does occur and might alter the subsequent response to parasite infection (Weil, Hussain, Ottesen).

Variable patterns of immune recognition and response to parasite antigens among different individuals may be important in determining both protective immunity and the different manifestations of human disease. Therefore, efforts to characterize the significant immunogens and allergens from the complex antigenic make-up of the parasite have been undertaken using a variety of immunochemical tools which allow the fractionation of radiolabelled parasite antigens which have been immunoprecipitated with human patient or immune rabbit antisera (Hussain, Anwar, Nash, Ottesen; see the annual report of Dr. Hussain).

In previous studies the prominence of immunoregulatory mechanisms in delayed hypersensitivity responses to chronic helminth infections has been emphasized. Since these infections also elicit high levels of specific IgE antibody (which is bound to mediator-rich basophils and mast cells) and since these cells are exposed in vivo, often continuously, to parasite antigens, we explored the reasons why allergic reactions (immediate hypersensitivity [IH] responses) are uncommon in chronic helminth infections. Our findings indicate that patients with chronic filariasis develop "blocking antibodies" in their sera which limit or contain IH responsiveness and that these antibodies are IgG directed against parasite antigens. They appear analogous to the blocking antibodies induced by the allergist's immunotherapy regimens for individuals with atopic diseases (Ottesen).

Schistosomiasis. Previous studies in our laboratory have defined the progressive loss of parasite antigen-specific cellular immune responsiveness (lymphocyte tranformation) in patients whose schistosome (Schistosoma mansoni) infections evolve from the acute to chronic stages. This "loss" has been found actually to reflect the development of specific immunoregulatory mechanisms in these patients. Suppressive elements have been identified (though not well characterized) both in the sera of chronically infected patients and among their adherent mononuclear cell populations derived from blood. Other mechanisms for the immunosuppression of lymphocyte function in these patients (which involve both T-lymphocytes and histamine) are currently under study, as are immunoregulatory mechanisms in immediate hypersensitivity reactions (Hofstetter, Ottesen).

Successful chemotherapy of S. mansoni infections can be achieved, but the effects of such treatment on patients' immune responses (and immunoregulatory mechanisms) have not been defined. Since these changes may be important in endemic areas in determining the effects of re-exposure to parasite infection and on the efficacy of immunization programs, we are currently following such changes in detail as part of a collaborative research-treatment field project in Puerto Rico (Hofstetter, Ottesen).

S. mekongi is a newly recognized schistosome parasite of man. Twelve Laotian refugees with this infection have been evaluated at the NIH with respect to their clinical and immunologic responses to infection. This study is the first detailed examination of such patients; our findings suggest that many of those same generalizations which have been made from studies of the host's immune response to S. mansoni infection (both delayed hypersensitivity and immediate hypersensitivity) are applicable to S. mekongi infections as well (Hofstetter, Barral, Nash, Cheever, Ottesen).

The Eosinophil. Eosinophils are prominant in the host response to allergens and helminth infections. These cells demonstrate autofluorescence of their cytoplasmic granules. With data derived from the spectral analysis of the fluorescence, a technique was devised to separate these cells from other leukocytes using the fluorescence-activated cell sorter. Sorted cells were highly purified, viable, and functional in vitro. The amount of fluorescence varied inversely with the absolute eosinophil counts of the patients under study. Efforts are now directed towards identifying the compound responsible for the fluorescence (Weil, Chused).

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1. Ottesen, E.A.: Modulation of the host response in human schistosomiasis mansoni I. Adherent suppressor cells which inhibit lymphocyte proliferative responses to parasite antigens. J. Immunol. 123, 1639-1644, 1979.

2. Ottesen, E.A.: Immunopathology of lymphatic filariasis in man. Springer Semin. Immunopathol. 2, 373-385, 1980.

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4. Ottesen, E.A.: Visceral larva migrans and other unusual helminth infections of man. In Principles and Practice of Infectious Diseases, Mandell, G.L., Douglas, R.G., Jr. and J.E. Bennett (eds.), Wiley & Sons, New York, p. 2191-2198, 1979.

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12. Cohen, S.G. and Ottesen, E.A.: "Eosinophils in Immune Function" in Oppenheim, J., Rosenstreich, D. and Potter, M. (eds.) Cell Biology of Immunity and Inflammation, Harvard Elsevier-North Holland, Inc. (in press).

13. Hiatt, R.A., Ottesen, E.A., Sotomayor, Z.R. and Lawley, T.J.: Serial observations of circulating immune complexes in patients with acute schistosomiasis. J. Infect. Dis. (in press).

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00094-21 LPD

PERIOD COVERED
September 30, 1979 to October 1, 1980

TITLE OF PROJECT (80 characters or less)
Studies on Entamoeba histolytica and other Parasitic Protozoa

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	L. Jacobs	Scientist Emeritus, NIH
	D. B. Keister	Biologist, LPD, NIAID
	E. C. Weinbach	Head, Physiology and Biochemistry Section, LPD, NIAID
	C. F. T. Mattern	Medical Officer, LPD, NIAID

COOPERATING UNITS (if any) American Type Culture Collection, Rockville, Md.; Hospital General, Centro Medico Nacional, I.M.S.S., Mexico City.

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Parasite Growth and Differentiation

INSTITUTE AND LOCATION
NIAID, Bethesda, MD. 20205

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SUMMARY OF WORK (200 words or less - underline keywords)

A basal medium incorporating defined nutrients in place of yeast extract was devised to facilitate further investigations of nutritional requirements of Entamoeba histolytica. Addition of bile extract to TYI-S-33 medium was essential for growth of one strain of Giardia lamblia and contributed to support of three other strains. A neonatal mouse model was developed for study of G. lamblia infections. Axenically cultivated trophozoites inoculated intragastrically through the abdominal wall produced infections which persisted for three weeks and were accompanied by cyst formation. Drastic modification of a cooling-rate schedule widely used for freeze-preservation of cells led to increased recoveries of E. histolytica stored in liquid nitrogen vapors. A simple inexpensive freezing device was highly effective for cryopreservation of E. histolytica, G. lamblia and Trichomonas hominis. Significant reductions in serum iron, total iron-binding capacity, and percent saturation of transferrin were found in 50 adult male patients acutely ill from amebic liver abscess in a Mexico City hospital study. These findings indicate that the disease is accompanied by changes in iron status similar to those in patients with acute bacterial or fungal infections. Studies to develop a means of inducing encystation of E. histolytica in axenic cultures were initiated.

Project Description:

The objectives are four fold: 1) to develop and refine techniques for the cultivation of lumen dwelling protozoa, especially Entamoeba histolytica and Giardia lamblia; 2) to determine their nutritional requirements in axenic in vitro systems and to define the physical and chemical conditions for optimal growth; 3) to study the mechanisms of their pathogenicity; 4) to devise or improve existing methods for the freeze-preservation of these parasites at cryogenic temperatures and to study mechanisms involved in resistance to freezing injury.

1. Defined Medium for Axenic Cultivation of Entamoeba histolytica: - The approach to developing a defined medium has been one of replacing each of the three undefined components of TYI-S-33 medium, i.e., Trypticase (casein digest), yeast extract and bovine serum, with groups of related defined nutrients know to be present in these components.

The yeast extract (YE) was replaced with a mixture of purines, pyrimidines, adenine nucleotides, B vitamins and an ammonium moiety which was supplied by one of the following compounds, urea, histidine, glutamate, or a simple ammonium salt. Current investigations have focused on improving the growth promoting effects of this replacement mixture. One mMolar concentrations of adenine, guanine, cytosine and uracil supported indefinite subculture of the amebae (strain 200:NIH). Increasing each of these bases in proportion to the levels present in YE was accompanied by a corresponding stimulation of growth. Each base could be replaced with its corresponding nucleoside, but growth was not improved. Of the three adenine nucleotides present, adenosine 5' monophosphate (AMP) was the most critical. As a result of these studies a basal medium (114) was devised to facilitate further investigations of additional defined nutrients of E. histolytica.

Gelysate, a pancreatic digest of collagen, when added to medium 114 stimulated amebal growth, but could not replace Trypticase even when fortified with amino acids in which it was comparatively low. Mixtures of essential and non-essential amino acids employed in defined media for other parasitic protozoa could not replace Trypticase. However, their addition in the presence of Trypticase improved growth. Single additions of the polyamines spermine, spermidine and putrescine, and the mitogenic proteins Epidermal Growth Factor and Fibroblast Growth Factor did not effect growth. Addition of the tripeptide Glycyl-L-Histidyl-L-Serine, while it did not stimulate multiplication, improved the appearance of the amebae markedly and stablized the cultures permitting more uniform growth patterns.

Attempts to replace Trypticase with defined nutrients will continue. Special attention will be given to the use of mixtures of amino acids with selected natural occurring and synthetic peptides. Studies on defining serum will be initiated (Diamond, Cunnick).

2. Axenic Cultivation of Giardia lamblia: - Bile supplemented TYI-S-33 medium has proved superior to other media for axenic cultivation of G. lamblia (Meyer's HSP-1 and HSP-2, Diamond's TP-S-1). Four strains have been maintained in it for periods ranging from 6 to 14 months.

The bile appears to be the crucial ingredient. A new strain isolated in this laboratory was found to be dependent upon its presence for survival. Studies to identify the essential components of bile have been unsuccessful. Neither glycine, taurine, cholic or chenodeoxycholic acids singly or in combination appear to be responsible for the growth stimulating effect. Further attempts to identify these factors will be made (Keister).

3. Animal Model for Study of Giardia lamblia: - Giardia lamblia infections were established in neonatal pathogen-free mice using axenically cultivated trophozoites. Inoculations were made intragastrically by passing a hypodermic needle through the abdominal wall, obviating laparotomy. Infections were produced with as few as 10 trophozoites and persisted approximately three weeks. Cysts recovered from feces exhibited typical morphology and were capable of producing infections when inoculated in the same manner as the trophozoites. To-date, infections have been obtained with parasites from four human isolates. The advantages of this model over others which use cysts obtained from feces of infected patients are that trophozoites are readily obtained in large numbers from culture, are free of contaminating fecal material and represent a more consistent population for study. Future investigations will be directed toward precise characterization of the infection (Keister, Mattern).

4. Cryopreservation Studies: - The recovery of viable E. histolytica immediately after thawing of freeze-preserved specimens was increased substantially by modifying the cooling rate schedule to obtain rapid cooling of the sample (5 to 8° C/min) between room temperature and the latent heat of fusion zone and ultra-rapid removal of latent heat so that the sample was not exposed longer than 60 sec. to the high temperatures.

Certain polyamines have the capacity to stabilize membranes. In trials to minimize loss of cell membrane integrity, a common result of freezing injury in E. histolytica, the organisms were cultured and frozen in the presence of one of the following polyamines: spermine, spermidine and putrescine. No enhancement in recoveries of viable amebae was obtained. Future investigations: The temperature at which a sample after transition from the liquid to the solid phase is taken and plunged into liquid nitrogen (plunge point) is critical for some protozoa. Selected plunge points will be examined for their possible effects on E. histolytica (Diamond, Daggett, Gillin, Keister and Cunnick).

The elaborate controlled-freezing equipment, so successfully employed for cryo-preservation of lumen dwelling protozoa, is beyond the means of all but the most affluent laboratories. In view of the need for simple less expensive, but effective freezers, the potential of the inexpensive BF-5 Biological Freezer (Union Carbide) was investigated. Axenically cultivated trophozoites of 12 strains of E. histolytica, 4 strains of G. lamblia, and 2 strains of Trichomonas vaginalis were preserved with this device. A cryopreservant consisting of DMSO, a simple sugar, and TYI-S-33 medium was used. The source of the coolant was the vapors evolved from liquid nitrogen contained in LD 30 or LR 30 refrigerators (Union Carbide). Cooling was controlled by the depth the BF-5 was inserted into the refrigerator and regulation of the level of the liquid nitrogen. Excellent recoveries of the three species were obtained 10 months after preservation (Keister).

5. Studies on Encystment of E. histolytica Under Axenic Culture Conditions: - Cysts of E. histolytica which transmit the infection to new hosts, do not form under axenic conditions. In contrast, encystment occurs more or less regularly in ameba-bacteria (A-B) culture systems and can be induced en masse. Methods devised for producing cysts in axenic cultures of the related E. invadens and certain free-living amebae are of no value when applied to E. histolytica. Therefore, in addressing the problem in axenic cultures our approach has been to attempt reproduction of those conditions under which encystment is known to occur in A-B cultures. In this system, a period of starvation induced by carbohydrate deprivation, followed by a short period of intensive multiplication upon carbohydrate repletion, leads to mass encystation.

Axenically cultivated strain 200:NIH amebae when deprived of glucose (normally added to TYI-S-33 medium) survived at low levels for several subcultures presumably by utilizing carbohydrate present in the yeast extract and serum. Addition of glucose resulted in rapid multiplication, but not encystment. In current studies the amebae are grown in the partially defined medium 114 which is free of yeast extract and does not support growth in the absence of added glucose. Carbohydrate starvation can be controlled precisely. After extended serial subculture in the presence of the lowest level of glucose consistent with minimal growth, the amebae will be returned to medium replete with glucose, hopefully encystment will ensue. Since, it is known that the composition of the bacterial flora in A-B cultures can influence cyst production, the effects of adding sterile extracts of selected bacteria to the system described will also be examined.

During a recent visit by the Principal Investigator to the Weizmann Institute of Science in Israel his attention was called to the presence of round bodies in axenic cultures of 200:NIH which were presumed to be cysts. Examination revealed these bodies to have all the morphological features of cysts except for the absence of distinct cyst walls. Similar structures have not been observed in cultures grown at the NIH. Using the Israeli substrain of 200:NIH attempts were made in this laboratory to reconstruct the conditions under which the formation of the cyst-like bodies occurred. Limited success was achieved, but only with amebae grown in cultures brought back directly from Israel and amebae taken from the first subculture made at the NIH. Comparative studies of the NIH stock cultures and the Israeli substrain revealed initial differences in growth patterns which have steadily narrowed upon subculture, the latter approaching the patterns of the former. We believe the inability to produce the cyst-like structures with the Israeli substrain after subculture in our laboratory is related to the changing patterns of growth. A second set of cultures have been brought from Israel. After one subculture to build up the stocks the amebae were frozen in an effort to preserve their original traits. Samples will be withdrawn from the cryo-bank one at a time and used to produce these cyst-like forms which may be aborted cysts (Diamond, Keister, Jacobs).

6. Iron, Nutritional Immunity and Amebic Disease: - Acute infectious diseases of bacterial and fungal origin are characterized by a lowering of the host's serum iron (SI) levels, total iron binding capacity (TIBC) of serum, and the percent saturation of transferrin with iron. These changes in iron status are believed to be part of the host's defensive mechanisms and serve to deprive the invading organism of iron, an essential nutrient. To determine whether similar responses were evoked in acute amebic disease, SI, TIBC and percent saturation of transferrin were measured in 50 acutely ill adult males hospitalized for amebic liver abscess during 1977 at the Hospital General, CMN, Mexico City. Significant reductions were found in each of the parameters investigated compared to healthy adults, a pattern compatible with those of other infectious diseases. Future investigations: This study has been phased out. Analysis of data on female patients with liver abscess and patients of both sexes with amebic colitis remain (Diamond, Pizzuto, Gallegos and Landa).

For results of collaborative studies with investigators in other sections within the Laboratory of Parasitic Diseases see the following annual reports: Project No. Z01 AI 00098-24-LPD, PI E. C. Weinbach; Project No. Z01 AI 00185-02-LPD, PI C. F. T. Mattern.

Publications:

1. Diamond, L.S.: Axenic cultivation of Entamoeba histolytica: Progress and Problems. Arch. Invest. Med. (Mex.). (in press).

2. Gillin, F.D. and Diamond, L.S.: Entamoeba histolytica and Entamoeba invadens: Effects of temperature and oxygen tension on axenic growth. Exp. Parasitol. 49: 328-338, 1980.

3. Gillin, F.D. and Diamond, L.S.: Colonal growth of Giardia lamblia trophozoites in a semi-solid agarose medium. J. Parasitol. 66: 350-352, 1980.

4. Gillin, F.D. and Diamond, L.S.: Attachment and short term maintenance of motility and viability of Entamoeba histolytica in a defined medium. J. Protozol. (in press).

5. Gillin, F.D. and Diamond, L.S.: Entamoeba histolytica and Giardia lamblia: Growth responses to reducing agents. Exp. Parasitol. (in press).

6. Gillin, F.D. and Diamond, L.S.: Entamoeba histolytica and Giardia lamblia: Effects of cysteine and oxygen tension upon attachment and survival in culture media. Exp. Parasitol. (in press).

7. Weinbach, E.C., Claggett, C.E., Keister, D.B., Diamond, L.S., and Kon, H.: Respiratory metabolism of Giardia lamblia. J. Parasitol. 66: 347-350, 1980.

8. Weinbach, E.C., Takeuchi, T., Claggett, C.E., Inoue, F., Kon, H. and Diamond, L.S.: Role of iron-sulfur proteins in the electron transport system of Entamoeba histolytica. Arch. Invest. Med. (Mex.) (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00097-22 LPD

PERIOD COVERED

October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Physiological and Cytochemical Pathology of Parasitic Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Teresa I. Mercado	Research Physiologist, LPD, NIAID

COOPERATING UNITS (if any) Ms. Alba Colon-Whitt, Laboratory of Streptococcal Diseases, NIAID and Dr. John Pisano, Hypertension-Endocrine Branch, NHLBI

LAB/BRANCH

Laboratory of Parasitic Diseases

SECTION

Physiology and Biochemistry

INSTITUTE AND LOCATION

NIAID, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The anti-trypanosome (lytic) factor (ATF) produced by *Pseudomonas fluorescens* initially reported last year was proved to be physiologically extracellular, not a consequence of lysis of the bacterial cells. Preliminary results indicate that the ATF is also active in vivo producing a marked drop in the parasite count. Studies on its chemical characterization are in progress.

Project Description:

The aim of our studies this year was to prove the physiologically extracellular nature of the anti-trypanosome factor (ATF), its effect on other flagellated organisms, its production by other bacterial species, its chemical characterization, and whether or not it was also active *in vivo*. Our first step was to employ a culture medium utilizing a minimal amount of protein, since our initial trials had indicated the factor to be protein in nature. A two-step procedure was used by which we grew the cells for 16 hours at room temperature in a defined medium containing tryptose, glucose, and salts, then separated them, washed three times, and incubated them for 4 hours in a (secretion) medium containing salts and only glucose as the organic solute. Centrifugation, filtration through Millipore, lyophilization, dialysis to remove salts, and re-lyophilization to concentrate the factor were employed. This experimental design was followed throughout to establish the characteristic logarithmic and stationary periods of growth and to time the occurrence of the factor. The sedimented cells resulting from these centrifugations were washed 3 times, sonicated, resuspended, and tested for lytic effect. Other bacterial species, e.g., *Bacillus subtilis*, *Escherichia coli*, and *P. aeruginosa* were also examined. The density of the cultures was determined with a Klett-Summerson colorimeter and they were grown on agar plates to determine the colony count. Dialysis tubing of different porosities were used. High performance liquid chromatography (HPLC) and Sephadex gel filtration were employed in preliminary purification trials. The flagellated species, *Leishmania brasiliensis*, *Trypanosoma rhodesiense*, *T. equiperdum*, and 2 other strains of *T. cruzi* were examined for their susceptibility to the ATF, and other bacterial substances (lipopolysaccharides from *Salmonella typhosa*, *S. typhymurium*, *E. coli*, and *Serratia marcescens* were assessed for lytic effects comparable to those of *P. fluorescens*. *In vivo* studies were performed employing mice inoculated intraperitoneally with the Tulahuen strain of *T. cruzi* and intramuscular injections of the ATF suspended in phosphate buffer. Blood smears, tissue imprints, and histologically processed tissues were stained with Giemsa.

Our findings disclosed the extracellular nature of the ATF; this was clearly indicated by its production during the logarithmic period of growth and its absence from washed, sonicated cells. Of the bacterial species studied, only *P. aeruginosa* produced lytic effects comparable to those of *P. fluorescens*, and endotoxins from the bacterial species mentioned above did not produce lysis of the trypanosomes. All the flagellated species studied were susceptible to the *P. fluorescens* ATF. HPLC and Sephadex gel filtration disclosed the occurrence of at least one active fraction, but the exact molecular weight and chemical characterization are currently being assessed. Preliminary estimates obtained with dialysis tubing filtration indicated a molecule between 6,000 and 12,000 daltons. Infected mice treated with 2 or 3 doses of ATF (25 to 200 μg protein) exhibited a rapid drop in the parasite count (within 24 hours) and liver and spleen of the treated animals did not exhibit the considerable necrosis and parasitic foci observed in the nontreated, parasitized controls. A marked release of white cells was observed both in infected and uninfected controls) which was always reproducible; this release subsided with time.

The immediate proposed course of this work is to purify the ATF and to repeat the in vivo experiments using the purified fraction. However, a more extensive elucidation of questions related to the cause of the drop in parasitemia in the treated animals, the marked leucocyte release, and the mechanism of action of the ATF would enhance our knowledge and understanding not only of the ATF but also of the enzymic function of the parasite membrane which is an important aspect of this project.

The observed relationship between a trypanosome and a bacterium provides another interesting example of microbial interaction. In view of the wide public health significance of Chagas' disease, these findings are also of potential importance in parasite chemotherapy and may aid further in our understanding of the cellular biology and physiology of flagellar motility.

Publications:

Mercado, T. I. and Garbus, J. 1979. Creatine phosphokinase isoenzymes and *Trypanosoma cruzi* infections. *Comp. Biochem. Physiol.* 64B:11-15.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00098-24 LPD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Biochemical mechanisms of energy metabolism in mammalian and parasitic organisms.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E.C. Weinbach	Head, Physiology and Biochemistry Section, LPD, NIAID
Other:	L.S. Diamond	Head, Parasite Growth and Differentiation Section, LPD, NIAID
	C.E. Claggett	Biological Laboratory Technician (Biochem.), LPD, NIAID
	D.B. Keister	Biologist, LPD, NIAID
	D.M. Dwyer	Research Microbiologist, LPD, NIAID

COOPERATING UNITS (if any)
Laboratory of Chemical Physics, NIAMDD (H. Kon); Metabolism Branch, NCI (D. Tschudy and P. Ebert).

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Physiology and Biochemistry

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
36/12	22/12	14/12

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies of aerobic energy metabolism in parasites and mammals were continued and devoted primarily to elucidating the sequence of electron transfer in the respiratory chains of Entamoeba histolytica and Giardia lamblia. Efforts were focused primarily on the iron-sulfur proteins in these parasites. The subcellular distribution and molecular properties of the Fe.S centers in E. histolytica differ from those observed in G. lamblia. Reexamination of L-serine metabolism in E. histolytica was necessary when it was found that the amebae no longer oxidize this amino acid. Detailed studies of this paradox have shown that the current strain cultivated in Diamond's new TYI-S-33 medium fail to respond to L-serine, whereas the old strain frozen since 1975 and recultivated in the old TP-S-1 medium, readily oxidize the amino acid as reported previously. Studies on the ATPase of Leishmania donovani kinetoplasts disclosed that these organelles readily hydrolyze ATP in the presence of Mg ion. Mammalian studies were continued on the effect of heme depletion on the respiration of cultured murine erythroleukemia cells, Walker ascites tumor cells, and isolated rat hepatocytes.

Project Description:

The object of this project is to conduct fundamental studies on the mechanisms of aerobic energy metabolism in mammalian and parasitic organisms. Currently these studies center primarily on identification and characterization of components of the respiratory chains of pathogenic, enteric protozoa, and elucidation of the pathway of electron flow to molecular oxygen. Other studies focus on bioenergetic phenomena in organelles isolated from protozoa, mammalian tissues and cultured tumor cells.

Tissues and cells are disrupted mechanically and subcellular fractions isolated by differential and gradient centrifugations. Cellular and subcellular constituents, enzyme activities, and metabolic pathways are determined, characterized and elucidated by enzymatic, chemical, radiochemical and physical methods. Changes in oxygen concentration are determined polarographically with the Clark electrode. Heme depletion in rat hepatocytes is accomplished pharmacologically by perfusion of 4,6-dioxoheptanoic acid.

Parasite studies: Continued investigation of the respiratory chains of Entamoeba histolytica and Giardia lamblia cultivated axenically in TYI-S-33 medium have revealed marked differences in the iron-sulfur electron transfer proteins of these parasites. Upon cell fractionation, the bulk of respiratory enzymes and electron carriers are found in the cytosol of E. histolytica, and in the particulate fraction of G. lamblia. Electron paramagnetic resonance (EPR) studies have shown the presence of multiple iron-sulfur centers in E. histolytica that exhibit characteristic "signals" after enzymatic reduction with NADPH. These centers appear to be of the ferredoxin-type that have low redox potentials. In contrast, the EPR signal obtained with G. lamblia becomes evident only in the oxidized state, and closely resembles the high potential Fe.S center of the Chromatium type. This difference in the properties of the Fe.S centers of the protozoa may be related to the large difference in their habitats (Weinbach, Claggett, Keister and Kon). In earlier work (1975) we first observed that L-serine markedly stimulated O_2 uptake of E. histolytica. Dr. Takeuchi, a visiting associate, extended this observation, and found that L-serine was converted to pyruvate, and that the O_2 uptake was due to oxidation of this keto acid. After these findings were published (1979) we found that L-serine no longer stimulated the respiration of E. histolytica. Detailed examination of this paradox is helping to resolve the riddle. Our earlier work was done with trophozoites cultivated in Diamond's original TP-S-1 monophasic medium containing Panmede, an ox liver papain digest. Subsequently, Diamond, et al. formulated a new medium TYI-S-33 containing yeast extract and iron salts which is superior in supporting growth of the axenized amebae. Currently, all of our studies are done with amebae cultivated in the new medium. These parasites do not oxidize L-serine, but specimens, which had been kept frozen and recultivated in the old medium, oxidized L-serine as we had originally observed. This is an interesting example of the effect of the cultivation medium on the biology of a parasite (Weinbach, Claggett, Keister and Diamond). Continued studies on the respiratory metabolism of Giardia lamblia have revealed the presence of multiple enzymes which catalyze the oxidation of reduced pyridine nucleotides (NAD(P)H). These are found both in the cytosol and particulate fractions of disrupted cells. The particulate enzyme tranfers electrons from NAD(P)H to molecular oxygen without the need for exogenous carriers, indicating intactness of the respiratory chain. Attempts to sub-fractionate the particles by differential or density gradient centrifugations showed that the enzymatic activity was not associated with discrete organelles. Oxidation of NAD(P)H in the soluble fraction was slow, but greatly increased upon addition of menadione or other electron acceptors. Studies with inhibitors of electron transport, disclosed differences in the properties of the soluble and particulate enzymes. The physiological significance of these results is that enzymes which oxidize reduced pyridine nucleotides are vital by providing a mechanism for the reoxidation of NADH generated during glycolysis by an organism that lacks both mitochondria and lactate dehydrogenase (Weinbach and Keister). Studies on mitochondrial bioenergetics were resumed this year by characterizing the ATPase of Leishmania donovani kinetoplasts. Similar to its mammalian counterpart, the enzyme is activated by Mg ion, and strongly inhibited by micromolar amounts of oligomycin. Paradoxically, the latent ATPase activity is not evoked by reagents that uncouple oxidative phosphorylation, but this may result from fragmentation of the organelle which occurs during their isolation. Such a conjecture is concordant with the absence of respiratory control reported last year (Weinbach, Claggett and Dwyer).

Mammalian studies: The collaboration with Dr. Tschudy, NCI, on the effect of heme depletion and biological oxidations were continued with isolated rat hepatocytes, and cultured murine erythroleukemia and Walker ascites tumor cells. The hepatocytes, isolated from control and drug-treated rats, exhibited respiratory control and other characteristics of oxidative phosphorylation. Rats treated with 4,6-dioxoheptanoic acid showed suppression of heme synthesis because of inhibition of the second enzyme in the heme biosynthetic pathway, but little diminution of respiratory metabolism. It appears that under conditions of heme shortage, the cytochromes are tenaciously preserved in the mitochondria of the hepatocytes (Weinbach, Tschudy and Ebert).

This project, which attempts to elucidate vital biochemical mechanisms common to all aerobic cells, has obvious implications for biomedical research in general (illustrated by the collaborative projects), and provides a rational basis for understanding bioenergetic mechanisms associated with parasites and parasitism. The parasite studies will be continued with emphasis on identifying the ultimate electron carrier that replaces cytochromes found in other eukaryotes. Experiments with electron donors that feed electrons into the cytochrome region of the mammalian respiratory chain, indicated that an oxidase is present in both *E. histolytica* and *G. lamblia*. We now have a new procedure utilizing affinity chromatography that promises to greatly facilitate the separation and purification of the soluble protozoan oxidoreductases. Once these enzymes have been purified, characterization of the Fe.S centers in regard to molecular organization and redox potentials can be achieved. The encouraging results of the preliminary experiments of ATPase activities of *L. donovani* will be extended with the collaboration of Dr. Dwyer. Mammalian work elucidating the effects of heme depletion and respiratory metabolism of various cultured cell lines and rat hepatocytes will be continued.

Publications:

1. Weinbach, E.C., Takeuchi, T., Claggett, C.E., Inoue, F., Kon, H. and Diamond, L.S.: Role of iron-sulfur proteins in the electron transport system of *Entamoeba histolytica*. Arch. Invest. Med. (In press).

2. Weinbach, E.C., Claggett, C.E., Keister, D.B., and Diamond, L.S.: Respiratory metabolism of *Giardia lamblia*. J. Parasitol. (In press).

3. Tschudy, D.P., Ebert, P.S., Hess, R.A., Frykholm, B.C. and Weinbach, E.C.: The effect of heme depletion on growth, protein synthesis and respiration on murine erthroleukemia cells. Biochem. Pharmacol. (In press).

4. Pazoles, C.J., Claggett, C.E., Creutz, C.E., Pollard, H.B. and Weinbach, E.C.: Identification and subcellular localization of catalase activity in bovine adrenal medulla and cortex. Arch. Biochem. Biophys. 200: 434-443 (1980).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00099-10 LPD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Biophysical Parasitology

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. S. Dvorak	Research Microbiologist, LPD, NIAID
Other:	M. S. Crane	Research Associate, LPD, NIAID
	G. A. Schmunis	Visting Scientist, UFRJ, Brazil

COOPERATING UNITS (if any) Applied Clinical Engineering Section, DRS; Television Engineering Section, CC; Instituto "M.F. Chaben," Argentina; and Instituto E. Chagas, Brazil.

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Physiology and Biochemistry

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

(A) Trypansoma cruzi clones, exhibit marked differences in isoenzyme profiles and growth kinetics which are stable and diagnostic of the source of the parasite. Organisms from all clones isolated thus far are capable of infecting vertebrate cells. Clones derived from chronic human infections have extremely long doubling times (about 8 days) resulting in an intracellular cycle of about 3 months duration. Clone-derived trypomastigotes are capable of infecting insects. The rate of transformation in the insect varies between clones. (B) The initiation of RNA synthesis in T. cruzi does not begin until completion of trypomastigote-amastigote reorganization. Therefore, the mRNA required for this transformation is present in the trypomastigote. After reorganizing, amastigotes synthesize the RNA required for intracellular growth and reproduction during the prereplicative lag period. (C) Clones of T. cruzi have been established which consistently undergo nearly 100% epimastigote-metacyclic trypomastigote transformation in liquid culture medium.

Project Description:

An interdisciplinary approach is being utilized to study the interaction of Trypanosoma cruzi with vertebrate cells and the basic cell biology/physiology of T. cruzi at several stages in its life cycle. Project emphasis has shifted away from the use of T. cruzi strains and toward the use of T. cruzi clones for many of the studies presently being pursued or projected. Five major topics are being studied: 1. Growth kinetics and isoenzyme characterization of T. cruzi clones derived from various sources; 2. Infectivity, growth and differentiation of T. cruzi clones in the insect, Dipetalogaster maximus; 3. Pattern of RNA synthesis of T. cruzi following infection of vertebrate cells; 4. Epimastigote-trypomastigote transformation in T. curzi clones; and, 5. Serum-mediated cytotoxicity to vertebrate cells.

1. Growth kinetics and isoenzyme characterization of T. cruzi clones. Trypanosoma cruzi clones were derived by single cell isolation from an acute human case of Chagas' disease, a human case of T. cruzi infection and from a naturally infected opossum. The organisms were established in biphasic medium, characterized enzymically and passaged into LIT medium and Bovine Embryo Skeletal Muscle (BESM) cell culture. Following routine serial passage through both LIT and BESM cell culture for 6 months, the isoenzyme profiles were again determined and found to be identical to the parent clones. Therefore, isoenzyme profiles are stable markers of T. cruzi clones. After 4 and 5 months of serial passage in LIT medium, the growth kinetics of the epimastigote stage of the parasite clones were determined. Clones derived from the sylvatic source had doubling times of 36-49.7 hrs.; clones derived from an acute human infection had doubling times of 117.2-133.7 hrs.; and clones derived from a human case of T. cruzi infection had doubling times of 169-208 hrs. An analysis of modal volume of 10^3 organisms at peak growth showed no overall correlation of this parameter to population growth rate. Preliminary data indicate that the growth rates of the vertebrate stage of the T. cruzi clones in BESM cell culture are similar to those occurring in LIT culture. These differences in growth rate might influence the course of Chagas' disease in man. Additional data from more cloned isolates will be required to establish the generality of this relationship.

2. Infectivity, growth and differentiation of T. cruzi clones in Dipetalogaster maximus. Tissue culture-derived trypomastigotes of selected clones were mixed with fresh blood and used to infect Dipetalogaster maximus via membrane feeding. A linear relationship was found between the

concentration of trypomastigotes present in the blood meal and the number of trypomastigotes ingested. The kinetics of growth in D. maximus of a clone derived from an acute human T. cruzi infection were statistically indistinguishable from growth kinetics observed in LIT medium. Epimastigote-metacyclic trypomastigote transformation did not occur over the 30-day time course of the study. However, under identical conditions, a sylvatic-derived clone underwent extensive transformation to metacyclic trypomastigotes. Removal through differentiation of replicative members of the parasite population resulted in a calculated doubling time which was significantly different from that observed in LIT medium.

3. Pattern of RNA synthesis of T. cruzi following infection of vertebrate cells. Human fibroblasts (Lesch-Nyhan) were utilized to study the pattern of RNA synthesis of T. cruzi during the early stage of the intracellular cycle. Due to the loss of the enzyme, hypoxanthine-guanine phosphoribosyltransferase, Lesch-Nyhan cells are unable to incorporate guanine into nucleic acids. Thus, the incorporation of guanine into RNA by the parasite is not masked by an equivalent event in the vertebrate cells. It was confirmed that extracellular trypomastigotes actively synthesize RNA. However, RNA synthesis by intracellular parasites does not begin until after trypomastigotes have reorganized to amastigotes. Therefore, the synthesis of new RNA does not appear to be required for the reorganization process. It is postulated that trypomastigotes contain or synthesize the mRNA required for amastigote transformation. However, if a trypomastigote does not enter a vertebrate cell, reorganization to an amastigote stage is followed by a "switch over" to synthesis of the mRNA required for epimastigote transformation and growth.

4. Epimastigote-metacyclic trypomastigote transformation. As previously reported, attempts to produce epimastigote-metacyclic trypomastigote transformation utilizing substances which induce transformations in vertebrate cells (e.g., cyclic AMP) have not been successful. Therefore, a new approach to the problem was taken. Clones of T. cruzi were selected from stocks exhibiting high rates of transformation to metacyclic trypomastigotes in LIT medium. Reproducible transformations can be induced rapidly in these stocks if they are established at a suitable density. Special nutrients or culture medium are not required. It is postulated that T. cruzi stocks which do not transform under identical conditions are "neoplastic" in that they have lost their capacity to exit the cell cycle and differentiate to trypomastigotes when presented with the appropriate stimuli.

5. Serum mediated cytotoxicity to vertebrate cells. Guinea pigs were infected chronically with one of three different strains of T. cruzi. Serum from these animals as well as serum from normal (uninfected) controls was tested for cytotoxicity to T. cruzi-infected or uninfected vertebrate cells (WI-38 or Guinea pig skin fibroblasts). No difference in cytotoxicity was found among the various combinations of infected vs chronic serum and heterologous vs homologous serum-cell systems.

Significance and Proposed Course of Program:

The study of host-parasite interactions at the cellular and subcellular levels is a topic of critical importance. The outcome of this interaction directly influences the physiologic state and survival potential of both the host and the parasite.

Research will continue to attempt to establish the generality of the correlation of isoenzyme type and growth kinetics to disease presentation in *T. cruzi* clones from diverse geographic areas. Initially, this will consist of characterizing 200-300 clones from well-documented *T. cruzi* infections in the Republic of Argentina. Using a recently installed fluorescence-activated cell sorter, studies will commence to determine if unique variant antigens can be identified with *T. cruzi* clones and if qualitative and/or quantitative differences exist in the K-DNA or N-DNA between clones or between morphologic stages in clones with high epimastigote-metacyclic trypomastigote transformation frequencies.

Publications:

1. Dvorak, J. A.: Trends in the use of *in vitro* cell cultures for *Trypanosoma cruzi* research. *in In vitro* Cultivation of Pathogens of Tropical Diseases. (in press).

2. Crane, M. S. and Dvorak, J. A.: Vertebrate cells express protozoan antigen after hybridization. *Science 208*: 194-196, 1980.

3. Crane, M. S. and Dvorak, J. A.: *Trypanosoma cruzi*: Interaction with vertebrate cells. DNA synthesis and growth of intracellular amastigotes and their relationship to host cell DNA synthesis and growth. *J. Protozool. 26*: 599-604, 1979.

4. Crane, M. S. and Dvorak, J. A.: *Trypanosoma cruzi*: Interaction with vertebrate cells. Pattern of RNA synthesis following infection of vertebrate cells. *J. Protozool.* (in press).

5. Dvorak, J. A.: *In vitro* studies of the interaction of *Trypanosoma cruzi* with vertebrate cells. *in* Proceedings of the International Congress on Chagas' Disease, p. 76, 1979.

6. Crane, M. S. and Dvorak, J. A.: Studies on DNA synthesis during the intracellular cycle of *Trypanosoma cruzi*: Host-parasite inter-relationships. *in* Proceedings of the International Congress on Chagas' Disease, p. A12, 1979.

7. Schmunis, G. A.: Host resistance to *Trypanosoma cruzi* infection. *in* Proceedings of the International Congress on Chagas' Disease, p. Q9, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00102-06 LPD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Pathogenesis of Disease Caused by Infection with Intracellular Parasites.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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SECTION
Cell Biology and Immunology Section

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
27/12	16/12	11/12

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

No obvious associations of HLA-tissue types with chronic Chagas' disease were found when over 400 individuals were typed at A, B and C-loci. DR (B-cell typing was similarly negative in about 300 total cases. Twelve isolates from patients with cutaneous leishmaniasis were tested in BALB/c mice via the foot-pad route to yield a spectrum of responses from no to large lesions. Numbers of viable organisms that could be cultured from biopsied skin lesions were estimated before and after treatment in patients with disseminated cutaneous leishmaniasis from the Dominican Republic. An ELISA test has been developed for antibodies in human strongyloidiasis using larval antigen from S. stercoralis and S. ratti.

Project Description:

Infection with the intracellular parasite, Trypanosoma cruzi, results in a serious chronic disease years later in some individuals. Biologic characteristics of the parasite that might contribute to disease, as well as immunologic features of the host response that contribute to disease are objectives of study. Similar parasite and host characteristics are under investigation in leishmaniasis. T. cruzi is studied mainly with cell culture techniques that permit quantitative assessment of infectivity of the organism for cells. A model of infection in the foot-pad of inbred mice is used for biologic activity of strains of leishmania. ELISA tests are used to measure antibody to parasite antigens.

1. HLA-typing in Chagas' disease. The expanded study of HLA-tissue typing of Brazilian patients with chronic Chagas' disease has now been completed. A total of approximately 425 individuals, with 35 to 50 in each of several chronic disease, control or family member groups were typed at A, B and C-loci. In addition, a total of about 300 patients, with between 25 to 35 in each study group were DRw-typed (B-cell) with frozen cells. No obvious associations of HLA with Chagas' disease were found, but a more sophisticated further analysis in continuing. Some new HLA specificities will be established. In addition, further observations are planned among this population of patients to correlate HLA background with outcome of chronic Chagas' disease. (Neva, Gusmao, Gam, Ward and Brazilian collaborators).

2. Cutaneous leishmanial strains in BALB/c mice. The ability of at least 12 strains of leishmania to infect BALB/c mice have been studied from patients with cutaneous disease. The foot-pad route of inoculation was used. The results showed a spectrum of response with some strains producing no lesions while others produced large lesions with abundant organisms within a few weeks. While factors, such as inoculum size, influence development of lesions, the main factor appears to be dependent upon the strain itself. This in-bred mouse model promises to be a useful tool for leishmaniasis research. (Neva).

3. Studies on disseminated cutaneous leishmaniasis. A variety of parasitologic observations were made during the evaluation of 4 patients with disseminated cutaneous leishmaniasis from the Dominican Republic. Strains from 3 of the 4 cases failed to produce significant skin lesions in BALB/c mice. Quantitative cultures from multiple skin biopsies in these patients revealed concentrations of 10^4 to 10^5 viable organisms per mgm of biopsied tissue. Local heat treatment in one patient and amphotericin in 3 of the cases resulted in rapid clinical improvement and concomitant loss or marked reduction in numbers of culturable viable organisms from the lesions.

4. ELISA test for antibodies to Strongyloides. Interest in developing a serologic test for strongyloidiasis was stimulated by the availability of S. stercoralis larvae in a chronically infected patient. For

comparison, antigens were also prepared from S. ratti and Nippostrongylus. The antigens are derived from third stage infective larvae grown in charcoal cultures, with larvae and/or eggs from infected animal or human feces as the starting material. Most sera from patients infected with S. stercoralis have been found to have antibodies to a water soluble extract of larval antigen by the ELISA test. The test appears to be reasonably sensitive and surprisingly specific, although it does cross-react with sera from patients with certain helminthic infections.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00103-13 LPD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Immunological Studies on Toxoplasmosis and Other Parasitic Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. N. Lunde	Research Zoologist, LPD, NIAID
Other:	E. A. Ottesen	Senior Investigator, LPD, NIAID
	T. E. Nash	Senior Investigator, LPD, NIAID
	T. J. Lawley	Medical Investigator, NCI

COOPERATING UNITS (if any)
Dermatology Branch, National Cancer Institute

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Host-Parasite Relations

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
18/12	13/12	5/12

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

By the use of fractionated antigens in ELISA it was possible to correlate length schistosome infection with humoral antibody response. One fraction reacted primarily with antibody in patients with acute or early schistosomiasis while another fraction reacted with sera from patients with chronic infections. An ELISA for the detection of immune complexes has been developed. Limits of sensitivity and 95% confidence limits have been established. Patients with lupus and rheumatoid with positive C1q binding also have positive ELISA C1q. The ELISA C1q is being used to detect immune complexes in sera from patients with filariasis and will be expanded to include schistosomiasis and malaria.

Project Description:

Emphasis has been focused on improving the enzyme-linked immunosorbent assay (ELISA) for serodiagnosis and study of host parasite immune responses in parasitic diseases. The ELISA is used to measure class specific antibodies to antigens prepared from different stages of the life cycle of *Schistosoma mansoni* and also to quantitate antigens from *S. mansoni*. ELISA has been employed to detect circulating immune complexes in sera from patients with auto immune diseases as well as in sera from patients with filariasis.

I. Differences in ELISA response to fractionated schistosome antigens in patients with acute and chronic schistosomiasis. Antigens were obtained from a DEAE fractionation of a TCA soluble-chloroform insoluble fraction of adult worms (Nash) and evaluated in an ELISA with sera from patients with acute or early schistosomiasis and from patients with chronic schistosomiasis. Two antigen fractions an anodic proteoglycan localized to the schistosome gut epithelial cells (GASP) and a phenol-sulfuric positive, 260nm non-absorbing fraction (PSAP) differentiated between acute or early and chronic schistosomiasis. The early infected patients had high levels of antibody to GASP and low levels to PSAP while in the chronic patients the antibody response to these antigens was reversed. (Nash)

II. An ELISA for the detection of immune complexes. Clq prepared from normal human serum by the method of Yonemasu and Stroud (1971) was adsorbed to micro titer ELISA plates and following serum incubation horse-radish peroxidase labeled anti-human IgG was used instead of conventional radio-labeled antibody in a solid phase assay for immune complexes. Sera from patients with auto immune disorders were obtained from T. J. Lawley (NCI) and sera from patients with parasitic infections obtained from E. A. Ottesen (LPD). While the assay is still in developmental stages, optimizing reagents and test conditions, the following information has been obtained:

1. The upper 95% confidence OD_{490nm} from 90 tests representing 28 normal human sera done at 6 different times over a period of 2 months was 0.57. The standard error was 4%. The standard error for a positive control serum with a mean OD_{490nm} of 1.21 on 11 occasions was 6%.

2. The limit of sensitivity using aggregated human IgG appears to be in the range of 10-20 ug/ml of undiluted serum.

3. Patients with rheumatoid and lupus who have positive Clq binding (Lawley) also have a positive ELISA Clq.

4. Sera from patients with filariasis (Ottesen) are being evaluated and positive results have been found with some of this sera. Correlation with already completed Clq binding results have not been done as yet.

Proposed Course:

1. ELISA methodology using fractionated antigen, GASP, (Nash) will be evaluated for the detection of circulating antigen in acute schistosomiasis.

2. The ELISA for measuring immune complexes will be continued and expanded to include sera from patients with filariasis, schistosomiasis, malaria, and other parasitic diseases. It is the hope to eventually be able to separate and identify the antigens and antibodies in these complexes since they can be considered pathogenic mechanisms in parasitic diseases.

Significance to Biomedical Research:

Application of ELISA for multipurpose seroepidemiological and clinical studies is being contemplated in areas endemic for malaria, trypanosomiasis, and schistosomiasis by the World Health Organization. The measurement of antibodies to specific materials in parasitic infections or diseases can yield information not obvious by the measurement of antibodies to crude materials. In fact, humoral response to the two antigenic fractions correlated with an important clinical parameter, length of infection. With the measurement of antibodies to a panel of antigens it may be possible to characterize patients or populations by serologic means.

Circulating immune complexes play an important part in the pathogenesis of several human diseases. In chronic parasitic diseases where large amounts of parasitic antigen, corresponding antibody and complexes may be present the study of IC is an important step towards improving serology and in understanding the mechanisms of parasite infection and immunopathology.

Publications:

1. Lunde, M.N. and Ottesen, E.A.: Enzyme-linked immunosorbent assay (ELISA) for detecting IgM and IgE antibodies in human schistosomiasis. Am. J. Trop. Med. Hyg. 29: 82-85, 1980.

2. Wyler, D.J., Blackman, H.J. and Lunde, M.N.: Cellular hypersensitivity to toxoplasmal and retinal antigens in patients with toxoplasmal retinochoroiditis. Am. J. Trop. Med. Hyg. (In press).

3. Lunde, M.N., Edwards, S.J., Hoffman, G.L., Knight, R.A., Margolis, L. and Powers, K.G.: Translations of parasitological articles reported during 1978 and 1979. J. Parasitol. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00108-09 LPD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Studies on the Biology and Immunogenicity of Malaria Sporozoites

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. W. Gwadz	Research Entomologist, LPD, NIAID
Other:	R. S. Nussenzweig	Professor and Head, Division of Parasitology, New York University, School of Medicine

COOPERATING UNITS (if any)
New York University School of Medicine

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Malaria

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
9/12	3/12	6/12

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER
☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
The purpose of this project is to study the immune mechanisms involved in sporozoite-induced infections, and to develop protocols for immunization with sporozoite antigens. Studies underway are concerned with development of methods for immunizing rhesus monkeys with Plasmodium knowlesi sporozoites, and characterization of the structure, function and immunogenicity of surface determinants of sporozoites.

PHS-6040
(Rev. 10-76)

Sporozoite immunization

Rhesus monkeys immunized by intravenous injections of radiation attenuated P. knowlesi sporozoites of a strain of Malaysian (H strain) origin are resistant to subsequent challenge by bite of mosquitoes carrying H strain parasites. However, these same monkeys are susceptible to challenge with P. knowlesi sporozoites of a strain from the Philippines (P strain).

Rhesus monkeys do not respond uniformly to P. knowlesi sporozoite antigens. Monkeys on similar immunization schedules receiving the same numbers of sporozoites all do not produce protective antibodies. Investigation of histocompatibility types in rhesus monkeys are underway.

Characterization of sporozoite surface antigens

Monoclonal antibodies have been produced against P. knowlesi sporozoite surface antigens. Spleen cells from Balb/c mice immunized with P. knowlesi sporozoites were fused with NS-1 myeloma cells. The resulting hybridomas produce antibodies which react with intact sporozoites giving both circumsporozoite precipitin (CSP) reactions and immunofluorescense (IFA).

Proposed course:

1) Characterization of sporozoite surface antigens using metabolic labelling and hybridoma produced antibodies will continue.

2) Hybridoma-produced antibodies will be tested for sporozoite neutralization activity (SNA).

3) Hybridoma-produced antibodies will be transferred to monkeys to determine if protective immunity to sporozoite challenge can be conferred on recipients.

Significance:

A malaria vaccine effective against mosquito-injected sporozoites would be of great importance to human populations at risk in much of the tropical world. Characterization of the functional sporozoite antigens could lead to biological or biochemical synthesis and elimination of unreasonable dependence on mosquito-produced sporozoite antigens for vaccine development.

Publications:

1. Gwadz, R.W., Cochrane, A.H., Nussenzweig, V., and Nussenzweig, R.S.: Preliminary studies on vaccination of rhesus monkeys with irradiated sporozoites of Plasmodium knowlesi and characterization of surface antigens of these parasites. Bull. W.H.O. 57 (Suppl.1): 165-173 (1979).

2. Nardin, E., Gwadz, R.W., and Nussenzweig, R.S.: Characterization of sporozoite surface antigens by indirect immunofluorescense: detection of stage - and species - specific antimalarial antibodies. Bull. WHO 57(Suppl. 1): 211-217 (1979).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00149-05 LPD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Physiology of Anopheles mosquitoes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. Rosenberg	Staff Fellow, LPD, NIAID
Other:	R. W. Gwadz	Research Entomologist, LPD, NIAID
	R. F. Beach	Staff Fellow, LPD, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Malaria Section

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
43/12	18/12	25/12

CHECK APPROPRIATE BOX(ES)
☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies encompass the physiology, behavior, genetics, and vector competence of anopheline mosquitoes, the vectors of malaria. Techniques of transplantation and perfusion have been used to study the fate of sporozoites released from oocysts growing in both refractory and susceptible mosquitoes. Plasmodium knowlesi sporozoites produced in the refractory Anopheles freebroni will not invade the An. freeborni salivary glands but will invade those of the susceptible Anopheles balabacensis. Sporozoites which do not invade salivary tissue are destroyed equally quickly in both refractory and susceptible species. Growth and reproduction in mosquitoes and reduvid bugs has been diminished by feeding these insects blood containing antibodies to their gut secretions. Some functional immunoglobulin also traverses the gut barrier into the hemolymph. Isoenzyme and biological studies have helped separate two natural populations of An balabacensis that may be different species of unequal vectorial capacity. The genetic and physiological basis for vector competence is also being investiaged in An pharoensis and An gambiae.

PHS-6040
(Rev. 10-76)

1. The specific nature of barriers to the sporogonic development of malaria in refractory mosquitoes is unknown. Anopheles freeborni is a good host for most primate malarias but not for the simian malaria Plasmodium knowlesi. Female An. freeborni fed on P. knowlesi infected monkeys develop large numbers of oocysts which release apparently normal sporozoites, but there is no subsequent infection of this mosquito's salivary glands. We determined that sporozoites harvested from oocysts growing in An. freeborni could infect the salivary glands of a P. knowlesi susceptible mosquito, An. balabacensis. The possibility that a destructive factor in the hemolymph peculiar to the hemolymph of An. freeborni prevents sporozoites from reaching the salivary glands was discounted when perfusion experiments showed that sporozoites that were unsuccessful in entering the glands disappeared at a rate of 75%/day from both the susceptible and refractory mosquitos' circulation. Cross transplants of glands are being done to determine if An. freeborni glands are innately resistant to P. knowlesi invasion.

2. It is reasoned that blood-sucking insects fed on vertebrates immunized against one of the insects vital gut secretions or hormones could be adversly affected. Delays in each of the 5 molts and halving of egg production has been achieved by feeding Rhodnius prolixus, a vector of Trypanosoma cruzi, on guinea pigs immunized with secreting guts of the same species of bug. Similar success has not yet been attained with mosquitos fed on mosquito gut antiserum. Some antibody can pass functionally unimpaired through the insect gut epithelium into the hemolymph. Anti-toxoplasmosis Fab2 has been detected by flourescent microscopy in the hemolymph of both mosquitos and R. prolixus 24 hr after fed on a rabbit immunized with Toxoplasma gondii. Ingested antibodies to ecdysone and juvenile hormone, essential hormones which circulate in the hemolymph, have not so far affected the insects' fecundity or longevity.

3. There is increasing evidence that the widely distributed mosquito species called Anopheles balabacensis is actually a group of closely related species. Possible differences in vectorial capacity between these sibling species may explain inconsistent malaria endemicity in Southeast Asia, where An. balabecensis is the major malaria vector. We maintain two populations of "An. balabacensis": one, from Thailand, does not mate in captivity; the other, from Malaysia, does. Although the two populations are morphologically indistinquishable (except for the recently discovered difference in shape of a single pupal bristle) we have found wide isoenzyme divergence. Also, the larvae of the Malaysia population hatch bimodally in clean water, but synchronously in water in which larvae of the Malaysian, but not the Thai, population had previously been reared.

4. Susceptibility of mosquitos to malaria is genetically determined. Control of malaria may be possible by supplanting a susceptible mosquito species with a refractory strain of the same species. Lines of Anopheles pharoensis, the major vector in Egypt, and Anopheles gambiae, the vector in Africa, susceptible or refractory to Plasmodium knowlesi and P.

cynolmogi are being established. These lines will be used to isolate the determinants of susceptibility to these simian malarias, which closely resemble the human parasites.

Proposed Course:

1. Species specific receptors may account for the refractoriness of *An. freeborni* glands to *P. knowlesi* sporozoites. Future research will investigate this possibility. The ability of sporozoites which have already invaded one susceptible gland reinvading another is a related problem that will be studied.

2. Further work on the use of antibody as systemic insecticide will focus on increasing the yield of mosquito digestive enzymes for use as antigen.

3. Factors which control hatch synchrony in Malaysian *An. balabacensis* are contributed to the larval breeding site by the previous generation of larvae. Future work will attempt to isolate these factors and determine the ecological significance of the phenomenon.

4. The biology and genetics of *An. gambiae* and *An. pharoensis*, particularly as they relate to vector competence, will be explored.

Significance to Biomedical Research:

Only *Anopheles* mosquitoes transmit humanmalaria. Mosquito behavior, physiology, and vector competence are, however, poorly understood. We seek an understanding of certain critical and possibly vulnerable stages in the life cycle of these insects. Disruption of the mosquito's life, or of the parasite's life in the mosquito, must diminish malaria transmission and improve health in the tropics.

Publications:

1. Beach, R. F.: Mosquitoes: Biting behavior inhibited by ecdysone. Science, 205: 829-831 (1979).

2. Beach, R. F.: Physiological changes governing the onset of sexual receptivity in male mosquitoes. J. Insect Physiology (In press).

3. Rosenberg, R.: Ovarian Ecdysone controls blood meal retention in the mosquito *Anopheles freebroni*. J. Insect Physiology (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00160-04 LPD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Cell Biology of Entamoeba, Giardia and Other Parasitic Protozoa

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	F. D. Gillin	Senior Staff Fellow, LPD, NIAID
Other:	L. S. Diamond	Head, Parasite Growth & Differentiation Section, LPD, NIAID
	T. E. Nash	Medical Officer, LPD, NIAID
	P. D. Smith	I.P.A. Researcher, LPD, NIAID

COOPERATING UNITS (if any)
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Dr. Pierre Daggett, American Type Culture Collection

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Parasite Growth & Differentiation Section

INSTITUTE AND LOCATION
NIAID, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
23/12	12/12	11/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. Studies with a succinylated derivative of concanavalin A (S-Con A) indicate that Con A binding surface antigens of Entamoeba histolytica are involved in trophozoite attachment. Inhibition of amebic attachment by S-Con A was not due to agglutination or capping and was not produced by anti-amebic sera or other lectins.

2. Giardia lamblia and E. histolytica trophozoites were killed by thiol blocking reagents which remain outside of cells. Killing was prevented by cysteine or by bovine serum albumin. This is evidence for crucial thiol groups on the parasites' surface.

3. Our new, precise quantitative method of determining viability of G. lamblia and E. histolytica trophozoites in vitro was utilized in drug studies. The susceptibilities of these unrelated intestinal pathogens to a variety of drugs were similar. However, G. lamblia was more sensitive to quinacrine and less sensitive to several inhibitors of protein synthesis.

4. A new strain of G. lamblia has been isolated from a patient who had been treated unsucessfully with quinacrine and with metronidazole. In vitro, the organism was sensitive to both drugs.

PHS-6040
(Rev. 10-76)

Entamoeba histolytica and Giardia lamblia are important human intestinal pathogens. While both amebiasis and giardiasis are endemic in tropical areas, the latter is increasingly recognized as a problem in temperate areas, including the United States, where it is the most commonly identified waterborne enteric disease. We have been developing and exploiting new quantitative methodologies for study of complex biological processes such as trophozoite attachment, motility and interactions with host cells.

I. The role of Con A binding cell-surface antigens in attachment of E. histolytica.

Important interactions between parasites and host cells occur at the level of the respective cell surfaces. Several prominent surface antigens of E. histolytica trophozoites have been shown to be Con A binding glycoproteins. The cytochemistry and Con A-induced redistribution ("capping") of these components have been elegantly described. We have undertaken to elucidate their function. Physiological studies required a medium lacking serum and sugars, as these prevent binding of Con A. We recently developed such a maintenance medium, which supported apparently normal amebic attachment, motility and viability for 12 to 24 hr. Fluorescence microscopy showed succinylated Con A (S-Con A) to be bound normally to the ameba. This divalent derivative did not agglutinate the amebae, in contrast to the tetravalent native Con A.

In the presence of S-Con A (5 to 50 μg/ml), attachment of trophozoites to glass was inhibited. Addition of the Con A-specific hapten, α-methyl mannoside restored normal attachment. Other lectins tested neither bound to the amebae nor inhibited attachment. S-Con A did not induce capping or pronounced rounding of the amebae which might explain the inhibition of attachment. Moreover, the inhibition of attachment might be due to coating of the amebic surface by S-Con A. However, the effects of high titer sera from 6 patients with invasive amebiasis or from hyper-immunized rabbits on attachment were the same as control sera.

These studies indicate a function for a Con A binding subset of surface antigens in the attachment of E. histolytica trophozoites.

II. Evidence of thiols on the cell surface of G. lamblia and E. histolytica

The importance of L-cysteine in the cellular economy of both G. lamblia and E. histolytica has been shown in our earlier studies: Cysteine supported maximal growth and was required for trophozoite attachment to glass both in growth media and in defined maintenance media. Cysteine also acted as a reducing agent in protecting the parasites from the lethal effects of oxygen. In addition, cysteine was required for growth and attachment, even under N_2 atmosphere. These findings led us to postulate the existence of thiol (-SH) groups in the parasites which require the constant presence of exogenous cysteine and are crucial to parasite attachment and survival. Some of these thiol groups may be on the cell surface.

This hypothesis was tested by use of two groups of specific sulfhydryl-blocking chemicals. The first group of chemicals quickly penetrate all cells and are toxic. Trophozoites of E. histolytica and G. lamblia were killed by very low concentrations (1-10μ M) in less than 5 min. The parasites were protected by cysteine, but not by bovine serum albumin (BSA), which remains outside the cells.

The second group was comprised of -SH blocking chemicals which do not penetrate intact cells. They bind to -SH groups which are on or easily accessible to the cell surface; they are not generally highly toxic. However they did kill both parasites. BSA, as well as cysteine, protected both parasites from this group of reagents.

These studies support our hypothesis that G. lamblia and E. histolytica have crucial cell surface thiol groups.

III. Quantitative in vitro assessment of drug sensitivity.

Giardia lamblia and Entamoeba histolytica, while unrelated, have important similarities which are relevant to drug studies. To date, no in vitro drug study on axenic G. lamblia has been published.

We have developed a precise quantitative method which was needed for assessing effects of drugs on survival of these parasites in vitro. The new method-clonal growth of the parasites in semisolid media is quantitative, sensitive, and convenient. The ability of a cell to multiply is a stringent criterion for viability.

The susceptibilities of G. lamblia and E. histolytica to a number of antimicrobial agents were tested. The minimum lethal concentration range of metronidazole (2 to 4 μg/ml for G. lamblia) was similar to that determined by microscopic observation and subculture of organisms in liquid medium. Although the shapes of the time-kill curves differed, both parasites were killed rapidly by metronidazole: ∿ 50% in 6 to 7 hours at 2 μg/ml or in 1 hour at 10 μg/ml. L-cysteine decreased the susceptibility of both parasites to metronidazole, but exerted a lesser sparing effect on G. lamblia with quinacrine, emetine, and chloramphenicol. Effects of quinacrine upon parasite viability and attachment to glass were compared: inhibition of attachment was not a primary effect.

The susceptibilities of these unrelated intestinal pathogens to a variety of drugs were similar. However, G. lamblia was more sensitive to quinacrine and less sensitive to several inhibitors of protein synthesis. Both parasites were highly susceptible to cycloheximide, quinacrine, metronidazole, and emetine. Higher concentrations of paromomycin, bacitracin, tetracycline, or chloramphenicol were required for killing. Finally, the parasites were not killed by gentamicin, neomycin, kanamycin, kasugamycin, or mycostatin.

IV. Isolation of a new strain of G. lamblia.

Until recently, all in vitro studies on giardiasis have utilized one strain (Portland-1) of G. lamblia which has been cultivated axenically since 1976. After several unsuccessful attempts, we have isolated a new strain of G. lamblia from the duodenal fluid of a patient with symptomatic giardiasis. A mixture of gentamicin, kanamycin and mycostatin, as well as penicillin and streptomycin was used. We had previously found these antibiotics to be safe for use with the Portland strain. Growth of the parasite in early passages was dependent upon addition of a piece of fresh hamster liver to the cultures. This strain has been of great interest as the patient from whom it was isolated had been treated unsuccessfully with metronidazole and with quinacrine, but was apparently cured by a combination of the two drugs (see report of P. D. Smith). In vitro, we have found the new strain to be as sensitive as Portland-1 to each of these drugs. With both strains, the effects of quinacrine and metronidazole, in combination, were additive, rather than synergistic or antagonistic. This may have clinical relevance as neither drug alone is 100% effective. The new strain has been used for antigen in development of an Elisa assay for serum antibody in patients with giardiasis (See P. D. Smith report).

Future Studies:

The work performed to date has yielded much new and useful information concerning the behavior of the two parasites in relatively simple in vitro systems. These studies and the new techniques developed have also been aimed at facilitating future work with more complex biological systems. Some of the questions we plan to study are:

I. Chemotaxis, attachment:

A. Are these parasitic protozoa capable of sensing and responding to chemical stimuli? Appropriate motility media will be used to determine whether parasites move toward various cellular "targets" including isolated cultured cell colonies, primary intestinal epithelial cells or intestinal organ culture.

B. Is there specificity to parasite attachment? Do parasites attach preferentially to surfaces coated with various natural or artificial polymers or to different types of cell or tissue, compared with glass? If we do find parasite-host cell interaction, we will try to analyze it by enzymatic and chemical modification of whole cells and surfaces of both parasite and host cells.

II. Host defense:

We are interested in the role of serum antibody, as well as specific and non-specific secretory factors in host defense in giardiasis. In preliminary studies, we have begun to determine the effects of sera from patients and hyper-immunized animals, and partially purified secretory factors upon attachment and survival of G. lamblia.

Publications:

1. Gillin, F.D., and Diamond, L.S.: Entamoeba histolytica and Entamoeba invadens: effects of temperature and oxygen tension on axenic growth. Exp. Parasitol. 49: 328-338, 1980.

2. Gillin, F.D. and Diamond, L.S.: Clonal growth of Giardia lamblia trophozoites in a semi-solid agarose medium. J. Parasitol. 66: 350-352, 1980.

3. Gillin, F.D. and Diamond, L.S.: Attachment and short term maintenance of motility and viability of Entamoeba histolytica in a defined medium. J. Protozool. (In Press).

4. Gillin, F.D. and Diamond, L.S.: Entamoeba histolytica and Giardia lamblia: Growth responses to reducing agents. Exp. Parasitol. (In Press).

5. Gillin, F.D. and Diamond, L.S.: Entamoeba histolytica and Giardia lamblia: Effects of cysteine and oxygen tension upon attachment and survival in culture media. Exp. Parasitol. (In Press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00161-04 LPD

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Immunochemistry of Parasitic Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH

Laboratory of Parasitic Diseases

SECTION

Host-Parasite Relations Section

INSTITUTE AND LOCATION

NIAID, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
26/24	14/12	22/24

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Identification of relevant antigens in schistosomiasis and other parasitic diseases continues. The measurement of antibody responses to individual components of complex mixtures may reveal clinically relevant information obscured by responses to crude mixtures. Purification of a trichloroacetic acid (TCA-S-C) fraction of adult schistosomes led to the finding that the magnitude of antibody responses to specific antigens present in crude TCA-S-C correlated with clinically pertinent parameters whereas the responses to crude TCA-S-C failed to do so. Levels of antibodies to PSAP, a polydisperse glycoprotein, correlated with the egg excretion rate but the responses to gut associated proteoglycan (GASP) correlated with the acuteness of infection. Levels of antibodies as measured by a number of methods correlated well. Radioimmunoassays employing labelled antigens were more sensitive and specific than an ELISA test employing poly(L-lysine) coated plates. Excretory and secretory antigens of adult B. malayi have been identified by radiolabelling E-S products and using these labelled materials to measure antibodies in infected patients. Analysis of the mixture revealed a number of parasite antigens and some contaminating host products.

Identification of relevant antigens and responses to parasite antigens in human infections continues to be a major thrust of this laboratory. Substances responsible for antigenic activity in a trichloroacetic acid soluble (TCA-S-C) extract of adult worms were identified. Immunoglobulin G responses to crude TCA-S-C and fractions obtained following DEAE chromatography were measured using an ELISA test (Nash and Lunde) that employed microtiter plates coated with poly(L-lysine) and radioimmunoassays using radiolabelled antigens (Nash). Antibody responses to purified antigens correlated with clinically relevant parameters. Levels of antibody to purified gut associated proteoglycan (GASP) were highest in early infected patients and depressed in endemically exposed chronically infected patients. Responses as measured by both methods correlated with the IFA-IgG antibody levels to adult schistosome gut epithelial cells suggesting measurement to the same material. Another antigen(s) was identified, PSAP. This polydisperse glycoprotein had an apparent molecular weight between 130,000-98,000 daltons. Responses to this material correlated significantly with the egg excretion rate. Since TCA-S-C contained both antigenic substances, responses of all the patients reflected the levels of antibodies to both components obscuring the clinically pertinent correlations observed using GASP and PSAP. Radioimmunoassays were more sensitive and specific than the ELISA test.

Using radiolabelled antigen, an assay was developed to measure GASP that was capable of detecting from 1-10 ng/ml. No antigen was detected in the or sera of any patient. All patients' sera contained detectable antibody. The amount of antigen released by adult worm pairs maintained *in vitro* ranged from 140-270 ng/worm/24 hours. Studies are underway to measure PSAP.

Labelling of parasites and their excretory-secretory (E-S) products *in vitro* by incorporation of radioactive precursors continues. In addition to the other precursors mentioned in earlier reports, methionine was readily incorporated into both adult schistosomes and adult *B. malayi* and excreted as both large and small molecular weight products. Initially encouraging results were obtained demonstrating the presence of small molecular glucosamine containing E-S products in schistosome and microfilaria supernates following incorporation of N-acetylglucosamine. However, reproducibility was varied; analysis laborious and difficult (Nash).

Recently E-S products of adult *B. malayi* were labelled with I_{125} (Anwar, Hussain, Nash, Ottesen). Antibodies to these labelled E-S products are produced in human infections. Acrylamide electrophoresis demonstrated the presence of minimally 6-7 bands some of which are parasite antigens and other contaminating host proteins. Studies are underway to further define these antigens and their biological relevance.

Publications:

1. Nash, T.E.: Schistosoma mansoni: Pattern of release of secretory products. Experimental Parasitology. 48, 92-99, 1979.

2. Cheever, A.W., Duvall, R.H., Minker, R.G. and Nash, T.E.: Hepatic fibrosis in rabbits infected with Japanese and Phillipine strains of Schistosoma japonicum. Am. J. Trop. Med. Hyg. (in press).

3. Nasir-ud-din, Nash, T.E., Jeanloz, R.W., McArthur, J.W. and Gminskin, D.M.: Immunologically induced alteration in the morphology of the cervical mucus of Macaca radiata. Fertility and sterility. 32, 230-232, 1979.

4. Nasir-ud-din, Jeanlow, R.W.., Nash, T.E. and McArthur, J.W.: Cervical mucus glycoproteins in reproduction study of cervical mucus from Macaca radiata. In Glycoconjugates, eds. Shauer, R., Boer, P., Buddecke, E., Kramer, M.F., Vliengenthart, J.F.G. and Wiegandt, H. 548-549. George Thieme Publishers, Stuttgart.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00162-04 LPD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Biochemical Cytology of Host-Parasite Interactions in Parasitic Protozoa

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	M. Gottlieb	Guest Investigator†
	P. Pinto DaSilva	Head‡, Membrane Biology Section, LPP, NCI
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58/12	46/12	12/12

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Aspects of the cell biology and immunology of host-parasite interactions among several intra- and extracellular parasitic protozoa are being investigated. Leishmania sp. and Trypanosoma sp. are being used as models of intracellular and extracellular parasitism, respectively. Emphasis is placed upon: 1) determining some of the basic chemical and antigenic properties of parasite surface membranes; 2) ascertaining the nature and extent of the interactions of parasite surfaces with specific host cell types; 3) defining the basic mechanisms involved in the intracellular survival and multiplication of parasites within host cells; and 4) attempts to determine the means by which parasites circumvent host immuno-defense systems. Techniques employed in these studies include: subcellular fractionation, ultracyto- and immunochemistry, electron microscopy, affinity chromatography, polyacrylamide gel electrophoresis, lectin assays, radio isotope labeling, gel immunoassays, and in vitro cell culture.

Project Description and Objectives:

How parasites evade host immune responses, are nourished, multiply, and eventually destroy the host are questions being asked in this investigation. In as much as all initial interactions between host and parasite occur at the level of their respective cell membranes, and understanding of the nature and physiology of these membranes is essential. It is to this end that parasite surface membrane structure, chemical composition, antigenic nature, and physiology are being investigated specifically with regard to leishmaniasis and trypanosomiasis. Further, parasite interactions with specific host cell types and immune systems are being studied to ascertain the role of parasite surface membranes in infectious disease processes.

Methods Employed:

Techniques used include: 1) fine structure (transmission and scanning electron microscopy, ultrastructural cyto- and immunochemistry, freeze fracture and -etching methods, and autoradiography); 2) subcellular fractionation; 3) analytical and preparative polyacrylamide slab gel electrophoresis and isoelectric focusing; 4) antibody and lectin binding assays; 5) radio isotope labeling and autoradiography; 6) chromatography (gel permeation and affinity); 7) qualitative and quantitative gel immunoprecipitin assays; and 8) *in vitro* cell culture.

I. Isolation and characterization of subcellular components from *Leishmania* and *Trypanosoma*.

1. Structure, carbohydrate composition and asymmetry, and antigenic properites of surface membranes isolated from *Leishmania donovani* (Dwyer).

Pellicular membranes (PM) were isolated from *Leishmania donovani* promastigotes using methods previously devised in this laboratory. These membranes displayed a characteristic structural asymmetry (i.e. subpellicular microtubules remained attached to the PM inner lamina). Detergent solubilized PM were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Carbohydrate components in such gels were "stained" with 7 different lectin-fluorescein conjugates. Results of these studies indicated that the isolated PM contained a minimum of ∿24 glycoprotein constituents. These ranged in ∿ molecular weight (MW) from $15\text{-}90.5 \text{X} 10^3$ daltons. Of these 8 contained α-D-mannose, α- and β-linked galactose, N-acetylgalactosamine, N-acetylglucosamine and α-L-fucose indicating their side-chain compositional heterogeneity. One peptide component of ∿ MW $60 \text{X} 10^3$ daltons contained only mannose. The remaining 15 presumptive glycopeptides contained mannose plus at least one other sugar. A presumptive polysaccharide(s)/glycolipid(s) band was also present in such gels. This band(s) was specifically "stained" by all lectins used indicating its heterogeneity in carbohydrate composition. In conjunction with our previous fine structure localization studies using ferritin conjugated lectins, the present results suggest that the carbohydrate containing constituents are asymmetrically oriented in the PM external lamina.

Isolated PM were extracted with Triton X-100 (TX), Nonidet P-40 (NP), Lubrol-WX (LB), Octyl- and Decyl-D-Glucoside (OG, OD), and Zwittergent 3-14 (ZG). Dissolution of the PM bilayer occurred to varying degrees with the several detergents. The subpellicular microtubules (MT) remained insoluble following such treatments, and were retained in structurally and spacially intact MT-nexuses. In such nexuses, the MT were cross-bridged to each other at 24.1 nm intervals along their longitudinal axes. SDS-PAGE analyses demonstrated that all of the detergents were approximately equivalent in extracting the ∿ 40 representative PM petide constituents. Rabbit anti-PM sera were reacted against the various PM detergent soluble fractions in gel diffusion and immunoelectrophoresis (IEP) assays. Results of these assays indicated that Tx=OG>NP>DG>LB>ZG for retaining the antigenic reactivity of the extracted PM components. The PM-Triton extract (PM-TX) was used in all subsequent immunologic assays. Results of IEP and 2-dimensional IEP demonstrated that the isolated PM contained a minimum of 19-21 major antigenic components. In gel diffusion reactions, sera from patients with visceral leishmaniasis gave numerous strong precipitin lines against PM-TX indicating that these membrane antigens are recognized by the host's immune system during the course of infection. Moreover, these results demonstrate that certain membrane antigens are common between the amastigote and promastigote developmental stages (i.e. PM-TX was derived from promastigotes) of the parasite. Further, sera from *L. donovani* terminally infected hamsters gave a precipitin line of identity with Kala-azar patients' sera indicating that the infected animal recognizes the same parasite PM antigen as the infected patient. Sera from patients with a diffuse cutaneous form of leishmaniasis (i.e. from the Dominican Republic, *Leishmania* sp.?) also gave a single specific immunoprecipitin with PM-TX. Immunoprecipitates from the latter patients' sera and from *L. donovani* infected hamsters with PM-TX were analyzed via SDS-PAGE. A single parasite PM antigen was identified in such gels. It was a glycoprotein of ∿ MW of $8X10^4$ daltons and was localized on the PM outer lamina.

2. Cross-reactive surface membrane carbohydrate antigens of *L. donovani* and *Trypanosoma cruzi* (Dwyer and Gottlieb).

Antiserum (Tc-CHO-As) against a *Trypanosoma cruzi* surface membrane polysaccharide antigen (Tc-CHO) specifically agglutinated *Leishmania donovani* promastigotes (Ld-P). No agglutination occurred with Tc-CHO-As following its absorption with Tc-CHO, Ld-P, surface membranes (Ld-PM)- or polysaccharide (Ld-CHO) isolated from Ld-P. Specific binding of Tc-CHO-As on the Ld-P surface membrane was demonstrated by light and electron microscopy using fluorescein and ferritin conjugated antibody methods. As ascertained by indirect immunofluorescence, binding of Tc-CHO-As to living Ld-P at 0-4°C induced the movement (i.e., "patching" and "capping") of the cross-reactive Ld-P surface membrane ligands. Briefly fixed Ld-P treated identically gave similar, albeit less extensive, surface "patching" indicating the polysaccharide-like nature and apparent lipid linkage of the Ld-P surface ligands. Antigenic identity between Tc-CHO, Ld-CHO, and a Triton X-100 extract of Ld-PM (Ld-PMTX) was demonstrated in gel diffusion reactions against Tc-CHO-As. In reciprocal reactions, antiserum (Ld-PM-As) made against Ld-PM gave precipitin lines of antigenic identity between Ld-PM-TX, Ld-CHO, and Tc-CHO demonstrating the surface membrane origin of the Ld-P cross-reactive polysaccharide antigens. The Ld-PM-As also gave precipitin lines of identity between Ld-PM-TX, Ld-CHO, and polysaccharide fractions isolated from Leishmania tropica (Lt-CHO) and L. braziliensis (Lb-CHO). Further, sera from several Kala-azar patients gave similar precipitin lines of identity among Ld-PM-Tx, Ld-CHO, Lt-CHO, Lb-CHO, and Tc-CHO suggesting the clinical significance of these cross-reactive antigens. These results indicate that a common or cross-reactive polysaccharide-like antigen exists in the surface membrane of the various species studied. This antigen(s) may be analogous to those group-type antigens which have been used for the clinical serodiagnosis of various bacterial genera.

3. Localization and characterization of L. donovani Pellicular membrane enzymes (Gottlieb and Dwyer)

Studies have continued concerning the enzymes characteristic of the L. donovani surface membrane. Acid phosphatase (E.C. 3.1.3.2.) was characterized enzymically (pH optimum=5, fluoride sensitive, and substrate specificity determined). A 5 fold enrichment of the enzyme was obtained with isolated PM versus whole cell homogenates. The enzyme was localized in intact cells to the surface membrane and to lysosome-like vesicles intracellularly. Cytochemical enzyme reaction product was restricted only to the external lamina of isolated PM. A 5' and 3' nucleotidase were demonstrated enzymically on the surface membranes of living promastigotes. The specific activity of the 3' enzyme was 20 times greater than the 5' activity in isolated PM fractions. The pH optima for the 3' and 5' enzymes were 8.5 and 7.0, respectively. Only the 5' enzyme was sensitive to fluoride inhibition. The 3' enzyme had an absolute divalent cation requirement for activity and the following ions were effective: $Co^{+2} > Ca^{+2} > Mg^{+2} > Mn^{+2}$. Substrate specificity of the 3' enzyme was demonstrated: 3'AMP>3'UMP>3'GMP>3'CMP; no 3' deoxyribonucleotides were hydrolyzed. Cytochemically, both the 5' and 3' enzymes were uniformly distributed on the surface of intact cells and were localized to the outer lamina of isolated PM. These results suggest that the 2 nucleotidase activities are important in salvaging purine bases as the parasites require exogenous sources of these bases for growth. Further, in light of the fact that vertebrate host cells do not possess any 3' nucleotidase activity, it seems possible that toxic 3' nucleotide analogs or drugs conjugated to 3' nucleotides might prove to be highly specific leishmanicidal agents for the chemotherapy of this disease in the future. A Mg^{+2} stimulated

ATPase and adenyl cyclase have been localized cytochemically to the inner lamina (i.e. cytoplasmic surface) in isolated PM. Results of these studies further demonstrate both the biochemical and physiologic asymmetry of the parasite surface membrane.

4. Freeze-fracture and -etching of *L. donovani* PM (Pinto DaSilva and Dwyer).

Fine structure freeze-fracture and -etching studies of intact and isolated *L. donovani* PM were continued to further discern the supramolecular structure of these membranes. Intramembranous particles (IMP) in the membrane "P"-face appeared to be aligned with respect to the subtending inner lamina attached subpellicular microtubules (MT). Side arm cross-bridge structures were discerned periodically along the longitudinal axis of MT indicating that the MT might be attached to the PM inner lamina via peripherally oriented membrane constituents. Further, the substructure of MT isolated free of attached membrane via detergent treatment was determined using deep-etching procedures.

5. Isolation and characterization of *L. donovani* kinetoplast-mitochondria (Weinbach and Dwyer).

Studies on a kinetoplast-mitochondrion fraction obtained from *L. donovani* have continued. The terminal respiratory metabolism of these isolated organelles is continuing with regard to the characterization of their constituent enzymes (e.g. ATPase, succinic dehydrogenase, etc.) and terminal electron acceptors. For details concerning these studies see Dr. Weinbach's report (i.e. Z01 AI 0098-24 LPD).

6. Isolation and characterization of surface membranes from *Trypanosoma rhodesiense* (Finerty and Dwyer).

Surface membranes were isolated from *T. rhodesiense* bloodstream forms and characterized fine structurally. The surface coat is absent from such membranes. Immunologic studies concerning the antigenic composition of both the surface coat and isolated membranes are currently in progress.

II. Host-parasite interactions.

1. Expression of parasite antigens on the surface of human monocyte derived macrophages *in vitro* (Berman and Dwyer).

Leishmania amastigote-infected human monocyte derived macrophages have been used as a model of leishmaniasis. Immunologic investigations have focused on the presence of *Leishmania* antigen on the surface of such infected macrophages. Labelling with rabbit anti-amastigote serum in conjunction with either fluorescein or ferritin conjugated goat anti-rabbit IgG revealed that both *L. tropica*- and *L. donovani*-infected macrophages express *Leishmania* antigen on their plasma membranes. Such antigen(s) are apparently poorly represented in promastigotes of

Leishmania, and are not well labeled by serum from patients or hamsters infected with L. donovani.

2. Use of colloidal gold as a fine structure lysosomal label (Berman, Fioretti, and Dwyer).

Colloidal gold was synthesized and used as a fine structure marker for secondary lysosomes of infected cells both in vitro and in vivo. Using this label, amastigotes of L. tropica were demonstrated to reside and multiply within the host cell secondary lysosomal system of a mouse tumor macrophage cell line (i.e. strain P388D1) in vitro. Colloidad gold was also injected into the foot pads of BALB/c mice which were infected with L. tropica. Results of this study demonstrated for the first time that Leishmania also reside and multiply within the lysosomal system of macrophages in vivo.

3. Pharmacologic studies of Leishmania infected human macrophages in vitro (Berman).

85-100% of Leishmania in human monocyte derived macrophages were eliminated by achievable serum concentrations of anti-leishmanial agents presently in clinical use. Experiments to evaluate the anti-leishmanial activity of experimental anti-leishmanial compounds revealed that approximately half of the organisms in this model could be eliminated by possibly achievable serum concentrations or higher of Allopurinol. There was only modest parasite elimination by easily achievable serum concentrations of Ketoconazole or an 8-aminoquinoline (WR 6026), but virtually complete killing of Leishmania could be obtained with high concentrations of these drugs.

III. Antigenic variation in African trypanosomiasis: host immune response and cellular localization of antigens (Gardiner and Finerty).

Trypanosoma rhodesiense infections are being studied in mice with two major objectives: 1) To ascertain the effect of antigenic variation in African trypanosomiasis on various arms of the host immune response, and 2) to investigate possible methods of identifying individual antigens of trypanosomes such that their synthesis and export may be examined subcellularly. To this end, several clones of T. rhodesiense have been raised in various inbred mouse strains (i.e. CB A/N, Swiss nudes, II Nu, and CBA/N nudes). All of these strains have specific deficiencies in their immune response and trypanosome antigenic variation is be exained in them using various immunofluorescence methods. Individual trypanosome variants have been demonstrated to exist longer in mice having B-cell deficiences. This affords the opportunity of antibody and perhaps cellular reconstitution experiments and the effect of such restoration on immunocompetence and antigenic variation. Immunocytochemical methods at the fine structure level are currently being used to ascertain the cellular sites of synthesis, transport, and insertion of variant antigens on the surface membrane of bloodstream trypanosomes.

Proposed Course:

1. Various monospecific antibody, lectin, and radiochemical probes will be used further to qualitatively and quantitatively elucidate the nature of the intact *Leishmania* sp. and *Trypanosoma* sp. PM.

2. Specific antibody and lectin affinity chromatography and radiolabeling techniques will be used to preparatively isolate specific parasite PM constituents for their subsequent detailed chemical analyses (i.e., amino acid and carbohydrate composition).

3. Monospecific antibodies against *Leishmania* PM antigens will be used as probes to ascertain the presence and identity of parasite antigens on the surface of infected macrophages *in vitro*. Such antibodies might also be tested for their efficacy to suppress parasite growth and multiplication in cultured macrophages *in vitro*.

4. Lipid compositional analyses of the isolated *Leishmania donovani* PM have just recently been initiated in collaboration with Dr. Momtaz Wassef (Guest Investigator-LPD) and Dr. Edna Kaneshiro (IPA to LPD from the University of Cinncinati). The enzymology of lipid biosynthesis and degradation in such membranes will also be investigated.

5. Studies will be pursued concerning the nature and cellular origin of parasite exoantigens present in host circulation, and their possible role in the state of host immunoresponsiveness.

Significance:

Basic research concerning the molecular and structural composition of specific protozoan parasite surface membranes and their interactions with specific host cell types might provide a basis for future immunoprophylaxis programs and regimens and, or, a more rational approach toward the development of effective delivery systems for chemotherapeutic treatment of these diseases of man.

Publications:

1. Dwyer, D.M.: The recent advances in *in vitro* cultivation methods of trypanosomatids. *In* Hutner, S. (Ed.): *Protozoological Actualities*, Post-Congress Publication of the 5th International Congress of Protozoology. Lawrence, Kansas, Allen Press. pp. 184-186, (1979).

2. Berman, J.D., Dwyer, D.M., and Wyler, D.J.: Growth of *Leishmania* in human macrophages *in vitro*. *Infect. Immun.* *26*: 375-379, (1979).

3. Dwyer, D.M.: Isolation and partial characterization of surface membranes from *Leishmania donovani* promastigotes. *J. Protozool.* *27*, In Press.

4. Dwyer, D.M. and D'Alesandro, P.A.: Isolation and characterization of pellicular membranes from Trypanosoma lewisi bloodstream forms. J. Parasitol. 66, In press.

5. Gottlieb, M. and Dwyer, D.M.: Surface and plasma membrane associated phosphatases of Leishmania donovani promastigotes. In Slutzk, G. (Ed.): Biochemistry of Parasites. London, Pergamon Press. In press.

6. Dwyer, D.M.: Structural, chemical, and antigenic properties of surface membranes isolated from Leishmania donovani. In Slutzky, G. (Ed.): Biochemistry of Parasites. London, Pergamon Press. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00174-03 LPD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Culture, physiology and antigenic analysis of sexual and asexual erythrocytic malaria parasites

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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130/12	79/12	51/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Animals have been successfully vaccinated against sexual and asexual malaria parasites. In addition, Plasmodium falciparum, the major malaria of man, has been grown in culture for the production of asexual stages and gametocytes. The present study will 1) Improve culture conditions for the production of large numbers of gametocytes, gametes and merozoites of P. falciparum; 2) Study of the physiology of exflagellation, fertilization and invasion of red cells by malaria merozoites; 3) Characterize structure, function and immunogenicity of surface determinants on gametes and merozoites; 4) Evaluate gamete vaccines in model systems for identification of the best antigens and adjuvants. 5) Characterize red cell membrane of infected red cells.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

1. Use of cultured Plasmodium falciparum to study the production and maturation of sexual and asexual blood stages of the parasite. The work will include studies on controls of differentiation of the parasite stages and the identification and expression of antigens characteristic of each parasite state.

2. Attempts will be made to measure the natural variation between genetically unrelated isolates of P. falciparum with respect to various physiological and antigenic properties of the parasites. Variation will be analysed at a physiological and molecular level and also genetically by cross fertilization when it becomes possible to produce gametocytes infective to mosquitoes from cultured material.

3. Study of structure, function and immunogenicity of antigenic determinants on the surface of extracellular sexual and asexual parasites. This will include the use of monospecific antibodies produced by hybridomas and lectins for parasite component purification.

4. Study of immunogenicity of crude antigenic preparations of sexual and asexual stages in model systems.

5. Study changes in the membrane of malaria infected red cells.

Each objective is interrelated with the others: Culture will be needed for production of antigenic material for analysis of both sexual and asexual erythrocytic parasites. Structure and function of parasite determinants will be studied in the framework of parasite physiology. Successful immunization with whole parasite preparations will identify sources of more purified immunogenic materials.

Methods:

1. Continuous culture of red cell stages of Plasmodium falciparum; isolation of sexual and asexual stages; purification of merozoites and gametes.

2. Use of model systems: P. gallinaceum-chicken for study of gametes and gamete immunity; P. knowlesi-rhesus for study of mechanism of invasion and gamete immunity.

3. Use of polyspecific antibody to parasite antigens for immune precipitation; high-resolution gel electrophoresis and electrofocussing separation techniques; ^{125}I lactoperoxidase surface labelling; labelling of sugars and other components by various methods; general metabolic labelling of all parasite components; and lectin column purification of malarial antigens.

4. Monospecific antibody produced in hybridomas for analysis of parasite surfaces and isolation of surface determinants.

5. *P. falciparum* parasitized red cells with (K+) and without (K-) will be layered over human endothelial cells in culture and the percent adherence measured.

6. Use of membrane feeding to feed malaria infected blood to mosquitoes in the presence of substances, e.g. hybridoma antibodies, to be tested for their effect on infectivity of the parasites to mosquitoes.

Results of Research:

Asexual Parasites

1. The majority of animals immune to asexual infection with *P. knowlesi* whether by immunization with antigen in Freund's complete adjuvant or by repeated infection precipitate a particular malarial antigen by crossed-immunoelectrophoresis. Non-immune animals that were infected only one time or immunized with antigens in other adjuvants (e.g., Freund's incomplete adjuvant or BCG) did not precipitate this antigen.

2. ^{125}I-lactoperoxidase labelled merozoites give a characteristic profile on acrylamide gel electrophoresis. Trypsin-treated merozoites that can no longer attach to or invade red cells lose the high molecular weight labelled proteins (150,000 D and 105,000 D) with the appearance of new bands at 70,000 D and 62,000 D. Thus the high molecular weight proteins are candidates for receptor proteins.

3. Monoclonal antibodies have been developed which bind to the surface of merozoites by immunofluorescence and by ultrastructural studies of ferritin antibody coated merozoites. These antibodies agglutinate merozoites but only one has an effect on invasion. Three of these hybridomas precipitate from metabolically labbelled schizonts a single glycoprotein with a molecular weight greater than 200,000 D. Pecularly, surface labelled merozoites contain no protein above 150,000 D whereas schizonts contain this 200,000 D protein. It is possible that proteolytic digestion during labelling causes loss of the high molecular weight band on merozoites.

4. It is possible that invasion of red cells by merozoites involves enzymatic cleavage. Substrates (N-α-benzoyl-L-arginine-4-nitroanilide, azoalbumin, and p-nitrophenyl-glycosides) were not cleaved during invasion. However inhibitors of proteolysis (TLCK and TPCK) did block invasion.

5. Concanavalin A (Con A) agglutinates *P. knowlesi* merozoites and fluorescinated Con A fluoresces on the surface of merozoites. Parasite proteins of *P. knowlesi* metabolically labelled with ^{35}S-methionine bound to Con A columns and were specifically eluted with α-methyl

mannoside. It may be possible to partially purify membrane glycoproteins by this method.

6. Previously we showed that proteolytic enzymes has no effect on invasion of rhesus red cells by _P. knowlesi_ merozoites. We have now tested papain and a mixture of various glycosidares and have failed to reduce invasion with these enzymes. Invasion by _P. falciparum_ is also unaffected by glycosidases, other than a 50% reduction previously reported for neuraminidase.

7. Sera from Gambians who are immune to _P. falciparum_ cross-react with surface determinants on _P. knowlesi_ merozoites. However, these antibodies do not block invasion of red cells by _P. knowlesi_ merozoites.

8. Red cell membranes of _P. knowlesi_ infected cells have a dramatic decrease in sialic acid and galactose labelling. Protein labelling have demonstrated pecularities of the infected red cell membrane.

Gametocytes and Gametes

1. Studies on gametocytogenesis. In rapid glowing cultures, few parasites are able to become gametocytes either spontaneously or in the presence of 1mM cAMP. As cultures enter a growth plateau, 10% of the rings convert to gametocytes spontaneously and almost 100% convert to gametocytes in the presence of 1mM cAMP. The ability to form gametocytes either spontaneously or in response to cAMP differs greatly according to the line or isolate of _P. falciparum_.

2. Hybridomas have been produced against surface determinants on gametes of _P. gallinaceum_. None of these monoclonal antibodies tested alone cause marked reduction in mosquito infectivity. Two of these antibodies, 11C7 and 10G3, synergistically cause marked reduction in infectivity. Either 11C7 or 10G3 alone mediate agglutination of male gametes in loose stellate bundles. The two together immobilize microgametes in thick rope-like bundles. Antibody 11C7 immunoprecipitates a protein of apparent MW of 225,000 D.

3. Most rhesus monkeys immunized over 3 years ago with a mixture of _P. knowlesi_ gametes in FCA continue to show high levels of anti-gamete antibodies. Upon challenge, all eight monkeys had virtually complete transmission-blocking immunity; mosquitoes fed on these monkeys did not become infected. Several animals with low pre-challenge antibody responses were apparently boosted by the challenge infection, did not infect mosquitoes and demonstrated high post-challenge antibody levels.

4. Gametocytes at the time of peak sexual and asexual parasitemias show a markedly reduced infectivity to mosquitoes. This reduction is not antibody mediated and is not related to transmission blocking immunity. Blood-borne factors associated with the erythrocytes appear necessary for infectivity of the sexual parasites to mosquitoes. Depletion of these factors at peak asexual parasitemia contributes to reduced infectivity.

Proposed Course:

1. a) Identify anti-merozoite monoclonal antibodies which block invasion of red cells in the *P. knowlesi* system.

   b) Determine mechanism of blocking - agglutination of merozoites or receptor blocking against attachment or junction formation.

   c) Isolate merozoite components as potential immunogens.

2. Identify receptor for *P. falciparum* and *P. knowlesi* including enzymatic treatment of receptor red cells, inhibition of invasion with sugars, glycoproteins and isolated Duffy components and testing of red cells lacking glycophorin.

3. Lectin purification of glycoproteins from malaria parasites (*P. knowlesi* and *P. falciparum*).

4. Study attachment of *P. falciparum* infected red cells to endothelial cells in culture. Determine the relation between presence of knobs and attachment, including the molecular basis for attachment.

5. Malarial antigens on red cells infected by *P. knowlesi* display serologic variation during successive recrudescence of parasitemia. We will attempt to identify the variant component on the red cells. First, variants will be cloned. We will attempt to label the variant protein by various labelling techniques and study these preparations by immunoprecipitation and two dimensional gels. Variant antigens will also be studied from metabolically labelled parasites.

6. We will attempt to identify changes in red cell components from infected cells and to determine the consequences of these changes in surface glycoprotein structure on the fate of schizont-infected cells *in vivo*.

7. Isolates of *P. falciparum* will be screened for their intrinsic ability to produce gametocytes of both sexes. Mature gametocytes will be used as a source of material for study of gametocyte and gamete antigens and to infect mosquitoes for genetic studies.

8. A range of properties of cloned isolates of P. falciparum will be studied in culture including production of gametocytes, knob production and adherence to endothelium, antigenic differences.

9. Monoclonal antibodies that block infectivity of P. gallinaceum gametes to mosquitoes will be used to isolate target antigens. Polyspecific antisera raised in immunized animals against these antigens will be tested for their ability to block mosquito infectivity. These antisera will also be tested for their ability to recognize an analogous class of gamete antigens in P. falciparum.

10. Attempts will be made to identify antigens characteristic of each sex and of the zygote using monoclonal and polyspecific antisera. Metabolic labelling will be carried out during gametogenesis to determine whether synthesis of antigens occurs during this process.

11. Long term antibody levels will be monitored in intact and splenectomized monkeys, and the role of challenge infections as a booster to transmission-blocking immunity will be investigated.

12. The factors in blood necessary for gametocyte infectivity will be identified and characterized.

Significance to Biomedical Research: The merozoite has been the focus of research for many years because it is the stage that invades red cells and thus is exposed to blocking antibody. Antibodies may block invasion by specifically binding to receptors at any point in the invasion sequence or by agglutinating merozoites. Of the multiple determinants on the merozoite surface, we are attempting to identify and isolate those involved in red cell attachment and those which may be targets for agglutinating antibody.

The membrane of the infected red cell, another target of the immune response, has gained malarial antigens which undergo changes in antigenicity during the course of infection. Recrudescence of parasitemia is associated with new variants. In addition, the red cell membrane has reduced amounts of its normal components such as spectrin, band 3 and other glycoproteins. In order to understand how these changes influence the survival and eventual elimination of the parasite, we are attempting to define components in the membrane of host origin and those malarial components exposed to the surface including the variant antigen. These studies may influence the choice of antigens for immunization and may suggest new chemotherapeutic approaches.

Whereas the asexual parasites described above are responsible for disease, the gametocytes infect mosquitoes and transmit the infection to others. Studies on blocking transmission by hybridoma antibodies have identified two hybridomas that synergistically block invasion in P. gallinaceum and have led to the isolation of the antigens from the gamete surface. It should be possible to identify similar antigens on the surface of gametes of P. falciparum. Although immunization with these antigens would probably not reduce disease in the immunized population, they may contribute to the eradication of the infection. In addition, the studies on P. falciparum gametocyte production in culture will not only provide a source of gametes but also of sporozoites.

Publications:

1. Miller, L. H., Johnson, J. G., Schmidt-Ulbrich, R., Haynes, J. D., Wallach, D. F. H., and Carter, R.: Determinants on surface proteins of Plasmodium knowlesi merozoites common to Plasmodium falciparum schizonts. J. Exp. Med. 151: 790-798 (1980).

2. Desowitz, R. S., and Miller, L. H.: A perspective on malaria vaccines. Bull. WHO. In press.

3. Howard, R., and Miller, L. H.: Invasion of erythrocytes by malaria merozoites: Evidence for specific receptors involved in attachment and entry. Ciba Symposium Publication. In press. 1980.

4. Kaushal, D. C., Carter, R., Miller, L. H. and Krishna, G.: Gametocytogenesis by malaria parasites in continuous culture. Nature. In press.

5. Gwadz, R. W., Carter, R. and Green, I.: Gamete Vaccines and Transmission-Blocking Immunity in Malaria. Bull. Wld. Hlth. Org. 57, (Supp. 1) (1979).

6. Carter, R. and Miller, L. H.: A Method for the study of gametocytogenesis by Plasmodium falciparum in culture: Evidence for environmental modulation of gametocytogenesis. Bull. Wld. Hlth. Org. 57, (Suppl. 1): 37-52 (1979).

7. Carter, R., Gwadz, R. W. and Green, I.: Naturally acquired immunity and antimalarial antibodies in relation to infectivity to mosquitoes in endemic Plasmodium falciparum. Report of Scientific Working Group on Immunology of Malaria, Panama, June 1979 (in press).

8. Carter, R. and Gwadz, R. W.: Infectiousness and Gamete Immunization in Malaria. Chapt. 17 in Malaria in Man and Experimental Animals. Ed. J. P. Krier, Academic Press (in press).

9. Wyler, D. J., and Miller, L. H.: Chapter 223: Malaria in Principles and Practices of Infectious Diseases, G. L. Mandell et al (eds). J. Wiley publisher.

10. Johnson, J. G., Epstein, N., Shiroiski, T., and Miller, L.H.: Factors affecting the ability of isolated Plasmodium knowlesi merozoites to attach to and invade erythrocytes. Parasitology. In Press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00184-02 LPD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Chemotherapy of Malaria: The Relationships Between Gametocytocidal, Sporontocidal and Hepatic Schizontocidal Drugs.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. W. Gwadz	Research Entomologist, LPD, NIAID
Other:	L. C. Koontz	Biologist, LPD, NIAID
	L. H. Miller	Head, Malaria Section, LPD, NIAID

COOPERATING UNITS (if any)
Cols. C. J. Canfield and D. Davidson, Division of Experimental Therapeutics, WRAIR, Walter Reeed Army Medical Center

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Malaria

INSTITUTE AND LOCATION
NIAID, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3/12	1/12	2/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have developed a simple rapid, inexpensive method for screening for anti-malarial drugs with activity against hepatic schizonts. Drugs with known activity against hepatic schizonts i.e. drugs which block relapses of certain human and simian malarias, appear to have gametocytocidal activity in the P. gallinaceum/Chicken/Aedes aegypti.

PHS-6040
(Rev. 10-76)

Project Description:

Gametocytes share certain similarities with latent exoerthrocytic hepatic schizonts in that both stages remain dormant until stimulated to undergo further development. The stimulus for gametocytes to complete development to the gamete stage is provided in the gut of a feeding mosquito. The stimulus for the exoerythrocytic parasite to complete development in the liver is unknown. The two stages also share a common sensitivity to 8-aminoquinoline drugs, particularly primaquine. Primaquine is the drug of choice for radical cures of relapsing malarias such as *P. vivax*. Therefore, drugs exhibiting gametocytocidal effects might produce a radical cure of a relapsing malaria by eliminating latent parasites from the liver. Alternatives to primaquine are desireable because of the drug's long course of therapy (14 consecutive days), because of unacceptable side effects in some individuals, and because of the high rate of drug failure against some strains of *P. vivax*.

Methods:

We have developed a simple screen using *P. gallinaceum*, in chickens which can detect drugs with gametocytocidal or sporontocidal properties by their effects on the subsequent development of the sexual stages of the parasite in *Aedes aegypti* mosquitoes. Gametocytocidal drugs act directly on gametocytes circulating within erythrocytes in the vertebrate hosts. Effects are irreversible and present as non-infectivity of the parasite to mosquitoes. Sporontocidal drugs affect the parasite after the parasite because extracellular and after initiation of gametogenesis within the gut of the mosquito vector. In this screen a candidate drug is administered directly to a chicken showing a patent parasitemia. One batch of mosquitoes is fed on the chicken just prior to drugging and another batch five hours later. The two groups of mosquitoes are then held 6-7 days, dissected, and their guts examined for the presence and number of developing oocysts. Drugs with gametocytocidal or sporontocidal properties affect parasite development in two ways 1) Oocyst numbers are significantly reduced, usually by over 95%; 2) Oocysts which do develop are less than 1/3 normal size at 7 days post-feeding and do not complete development. Drugs with blood schizontocidal properties have no effect on the development of the parasite in the mosquito.

Gametocytocidal and sporontocidal drugs can be further differentiated with a 2 step technique using membrane feeders. 1) Blood from a previously drug-treated chicken is washed, resuspended in normal plasma and fed to mosquitoes through a membrane. 2) Plasma from a drug-treated chicken is mixed with parasitized blood from an untreated bird and fed to mosquitoes through a membrane. Drugs with gametocytocidal properties affect the parasite in the circulation; washing (technique #1) does not restore gametocyte infectivity. Conversely, plasma from a drug treated chicken has no inhibitory properties (technique #2). Sporontocidal drugs affect the parasite only within the gut of the mosquito. Parasites washed, resuspended in normal plasma and fed to mosquitoes are infectious. Plasma from drug-treated chickens mixed with parasites from untreated chickens inhibits oocyst growth.

Results:

A series of coded compounds provided by the Division of Experimental Therapeutics, WRAIR, were tested in the *P. gallinaceum*/mosquito screen. After the code was broken we found that drugs effective in producing radical cures of *P. cynomolgi* in rhesus monkeys, i.e. drugs which act against latent hepatic schizonts, had clearly demonstrable gametocytocidal properties in our screen.

Drugs with sporontocidal properties were less clearly differentiated. Some of these, such as pyrimethamine, appeared to have both gametocytocidal and sporontocidal effects, although pyrimethamine does affect hepatic schizonts.

Proposed Course of the Project:

This project has been completed and will be terminated. Results are being prepared for publication.

Significance to Biomedical Research:

A substitute for primaquine, the only available hepatic schizontocide, is considered one of the major needs for malaria therapy. However, development of new drugs has been hampered by the lack of a simple, inexpensive screen capable of evaluating a wide range of candidate compounds. We feel that the *P. gallinaceum*/*Ae. aegypti* system may function as a primary screen for hepatic schizontocides, with a capacity for evaluating large numbers of compounds including classes of drugs not yet tested against the latent tissue phases of the malaria parasite.

Publications:

1. Koontz, L. C., Jacobs, R. L., Lummis, W. L., and Miller, L. H.: *Plasmodium berghei*: Uptake of clindamycin and its metabolities by mouse erythrocytes with clindamycin-sensitive and clindamycin-resistant parasites. *Exp. Parasitol*. *48*: 206-212 (1979).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00185-02 LPD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Pathogenic protozoa: Structure, Virulence factors and endogenous viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. F. T. Mattern	Senior Investigator, LPD, NIAID
Other:	W. A. Daniel	Biologist, LPD, NIAID
	P. C. Natovitz	Biologist, LPD, NIAID
	D. B. Keister	Biologist, LPD, NIAID
	L. S. Diamond	Head, Parasite Growth and Development Section, LPD, NIAID

COOPERATING UNITS (if any) Dr. B. M. Honigberg, University of Massachusetts; Drs. Michael R. Spence and John K. Frost, Johns Hopkins University; Dr. David Kobiler and David Mirelman, Weitzman Institute, Israel.

LAB/BRANCH
Laboratory of Parasitic Diseases

SECTION
Cell Biology and Immunology

INSTITUTE AND LOCATION
NIAID, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project involves the study of the biology of Entamoeba and Trichomonas. The amebal toxin study continues to attempt to purify the toxin and identify the nature of its lectin-like activity. The toxin is neutralized by purified IgG from sera of patients known to have had invasive amebaisis with a general antiamebal antibody response, but not by IgG from sera of control individuals. Studies on selecting and maintaining virulent subpopulations of relatively avirulent amebal strains has been completed after more than 2 years and is being prepared for publication.

Recently a sensitive animal model for Giardia lamblia infection has been found in the newborn mouse inoculated intragastrically.

A study of Tritrichomonas muris revealed excretion of many viable pseudocysts in the feces of pregnant and lactating hamsters and the early infection of the newborn. A major project on Trichomonas vaginalis in collaboration with a grant supported study at the University of Massachusetts and Johns Hopkins University has been initiated.

PHS-6040
(Rev. 10-76)

Project Description:

1. Entamoeba histolytica:

Studies on the lectin-like toxin of E. histolytica are continuing. A comparative study of the toxin and a hemagglutinating lectin from E. histolytica (Kobiler and Mirelman) has shown the two to share a number of properties, but also to differ to an extent that suggests the two are different substances. They are similar in respect to solubilization by sonication; stabilization by reducing agents; and inhibition by normal serum, IgG from patients with invasive amebiasis, and N-acetyl-glucosamine (NAG) containing macromolecules (chitin, peptidoglycan, IgA). They differ in solubilization by freeze-thawing, pH dependence, and inhibition by NAG-oligomers, fetuin, and gangliosides. (Kobiler, Mirelman, Mattern).

Toxin activity has recently been shown not be absorded from solution by consecutive passage on cell sheets; quite unexpectedly, the activity was found to increase with passage suggesting the removal of an inhibitor or some sort of toxin "activation". (Mattern, Natovitz).

In order to determine whether the toxin was antigenic in man, sera from 10 cases of amebic liver abscess which sera were known to have antiamebic antibodies and sera from 5 persons with no history of amebiasis were studied. The IgG fraction of each serum was separated by DEAE cellulose column, the purity of each verified by electrophoresis. The IgG fractions from the 10 sera from infected persons inhibited toxin activity at >1:32 dilution, the 5 control sera showed no inhibition. (Mattern, Natovitz, Keister)

Progress in purification of the toxin includes the stabilization of the toxin by the enzyme inhibitor, phenyl methyl sulfonyl fluoride, and by the reducing agent, 2-mercaptoethanol (Kobiler, Mirelman, Mattern).

Studies on the isolation of virulent subpopulations from relatively avirulent strains of E. histolytica have been completed after 2 years and are being prepared for publication. Alternate passage of E. histolytica in the new born hamster liver and in axenic culture with selection of amebae from lesions produced at limiting dilution provided the selection pressure for obtaining lines of 2 different strains one of which has maintained a 1000-fold increase in virulence over that of the parental strain for 2 years of axenic culture alone (Mattern, Keister).

2. Giardia lamblia:

Intragastic inoculation of the new born mouse has provided an animal model for infection with Giardia lamblia. Inoculation of 10 to 10^6 trophozoites established a self limited infection of about 3 weeks duration in suckling mice. The infection, produced to date by 4 axenically cultured strains, includes production of typical cysts by both light and electron microscopy. Intragastric injection of cysts verified their ability to excyst and produce viable trophozoites (Keister, Mattern).

3. Tritrichomonas muris:

Female golden hamsters, either in the last week of pregnancy or in the first weeks of nursing, excreted in their feces variable and unpredictable numbers of pseudocysts of T. muris.

Pseudocysts examined by electron microscopy showed internalization of the three anterior flagellae and the undulating membrane with its recurrent flagellum. The undulating membrane and associated marginal lamellæ were characteristic of T. muris. Pseudocysts slowly became motile after 2 or more hours of incubation in medium. These "excysted" trophozoites were also identified ultrastructurally as T. muris.

New born hamsters showed no infection with T. muris at 3 days of age, but by the 7th day essentially all were found to have infected ceca, concomitant with cecal enlargement and the appearance of adult-type feces.

This study has clarified several uncertainties: The feces contain pseudocysts (no cyst wall) rather than true cysts, pseudocysts are viable in culture medium, being capable of "excysting", and pseudocysts undoubtedly play a role in transmission of the infection to the newborn (Mattern, Daniel).

4. Trichomonas vaginalis:

A 3 year grant to B. M. Honigberg (Principal Investigator, Univ. of Mass.) and John K. Frost and Michael R. Spence (Johns Hopkins Univ., Coinvestigators) has been approved effective Aug. 1, 1980. This unit in the LPD will provide collaborative support for this project in order to evaluate new assays for virulence of T. vaginalis (new born mouse, tissue culture), search for plasmids or viruses of trichomonads, evaluate virulence factors such as toxins and attempt to select virulent trichmonad subpopulations.

Prior to the effective date of the grant, simplified methods of isolation, culturing, testing for sterility and cryopreservation of fresh isolates of T. vaginalis have undergone preliminary testing with the Hopkins clinical group. The use of Diamond's TPI-S-33 medium (pH 6.0) and a simple freezing procedure (both originally employed for T. vaginalis by D. B. Keister, LPD) seem feasible on the basis of 8 new isolates of T. vaginalis (Mattern, Spence).

Assays of virulence of T. vaginalis in a tissue culture system show considerable promise as do assays in the new born mouse (Mattern, Natovitz).

Publications:

1. Mattern, C.F.T., Keister, D.B., and Natovitz, P.C.: Entamoeba histolytica "toxin:" fetuin neutralizable and lectin-like. Am. J. Trop. Med. Hyg., 29, 26-30, 1980.

2. Mattern, C.F.T., Natovitz, P.C., and Keister, D.B.: Detection of antibodies against lectin-like "toxin" of Entamoeba histolytica in sera of patients with invasive amebiasis. Arch. Invest. Med. (Mex.) in press.

3. Nomura, S., Daniel, W.A., Fernandez, J.A., Pang, R.H., and Mattern, C.F.T.: Morphologic changes in the rabbit SIRC cell line induced by simian sarcoma-associated virus. Virology, in press.

Honors and Awards:

Dr. Mattern serves on the editorial board of the Journal of Protozoology and the Transactions of the American Microscopical Society.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00197-01 LPD

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Immune Recognition in Filariasis and Other Helminth Infections

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Rabia Hussain	Collaborative Researcher (IPA)
Other:	Eric A. Ottesen	Senior Investigator, LPD, NIAID
	Nughat Anwar	Visiting Fellow, LPD, NIAID
	Theodore E. Nash	Senior Investigator, LPD, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Parasitic Diseases

SECTION

Host-Parasitic Relations Section

INSTITUTE AND LOCATION

NIAID, Bethesda, Maryland 20205

TOTAL MANYEARS: 18/12	PROFESSIONAL:	OTHER:

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Highly sensitive radioimmunoassays have been developed to estimate μg/ml levels of both IgE and IgG classes of specific antibody response in the sera of patients with filariasis and other helminth infections. Immune responses to various antigens from each of the major stages of the parasites are also being analyzed in an effort to understand the pathogensis of the wide spectrum of clinical presentations seen in these infections. The antigens derived from each of the stages are being rigorously characterized both biochemically and immunochemically. Furthermore, the basis of cross reactivity among various nematodes and cestodes is also being studied by inhibition radioimmunoassays with a goal toward developing more specific immunodiagnostic reagents.

Project Description:

The major objective of this project is to characterize the host's humoral immune response to filarial infections and to correlate it with the wide spectrum of clinical presentations observed in endemic populations.

Identification and Characterization of Filarial Antigens. *Brugia malayi*, a human filarial parasite can be maintained in gerbils and has been shown to have sufficient cross reactivity with *Wuchereria bancrofti* to warrant its use to study bancroftian filariasis. The three main stages (adult, microfilaria and infective larvae L_3 stage) to which the host is exposed for varying periods of time can be studied. In addition, there are two aspects of each stage which are especially likely to be recognized by the host: the surface and the secretions. The uniqueness of antigens present in each of the various stages is being rigorously studied immunochemically using radioimmunoelectrophoresis and SDS polyacrylamide gel electrophoresis in combination with autoradiography. Filarial antigens which are predominatly recognized by various immunoglobulin classes, especially IgE and IgG, are being identified and characterized using a combination of radioimmunoelectrophoresis and SDS polyacrylamide gel electrophoresis. Our initial studies show that the excretory-secretory antigens are extremely potent immunogens for both the IgE and IgG classes of immunoglobulin and both surface and metabolic products have been identified in this group of antigens. Further studies with other stages of the parasite are in progress.

Quantitation of Specific IgE and IgG Levels in an Endemic Population. A highly sensitive solid phase radioimmunoassay (SPRIA) was developed using *Brugia malayi* adult antigens. A high titered serum from a patient with tropical eosinophilia was prepared as a standard by quantitating the amount of specific IgE using a combination of reciprocal plot and IgE depletion analysis. Specific antifilarial IgE in this serum was determined to be 27 μg/ml (out of a total of 193 μg/ml). The sensitivity of the assay ranged from 0.6-600 μg/ml of serum, and competing IgG was shown not to affect the sensitivity of the SPRIA. Sera from a large endemic population (Cook Islands) were analyzed against this reference serum. The levels of specific IgE in the population ranged from 4-600 μg/ml with a mean of 47 μg/ml (N = 63). Interestingly, exposed individuals without any clinical presentation had significantly higher specific and total IgE titers than those individuals without clinically documented infection. This observation is consistent with the notion that specific IgE antibodies may play a role in protective immunity.

Work is in progress to determine the levels of specific IgG to both adult and excretory-secretory products of *Brugia malayi* in an effort to correlate the clinical and pathologic features of the disease in these patients with specific types of reaginic antibody responses.

Publications:

1. Marsh, D. G., Hsu, S. H., Hussain, R., Meyers, D. A., Friedhoff, L. R., and Bias, W. B. Genetics of human immune response to allergens. J. Allergy and Clinical Immunology. 65:322-332, 1980.

2. Marsh, D. G., Belin, L. Bruce, C. A.. Lichtenstein, L. M., and Hussain, R. Rapidly released allergens from short ragweed pollen. I. Kinetics of release of known allergens in relation to biological activity. J. Immunol. (in press)

3. Hussain, R., Norman, P. S., and Marsh, D. G. Rapidly released allergens from short ragweed pollen. II. Identification and partial characterization. J. Allergy and Clinical Immunology (in press).

4. Hendricks, A. A., Calvin, L., Elfenbein, G. J., and Hussain, R. Erythema annulare centrifugum associated with ascariasis. Arch. of Dermatology (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00198-01 LPD

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Host Immunological Response to Giardia lamblia Infection

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. D. Smith	Guest Investigator, LPD, NIAID
Other:	T. E. Nash	Senior Investigator, LPD, NIAID
	F. D. Gillin	Senior Staff Fellow, LPD, NIAID
	D. B. Keister	Biologist, LPD, NIAID
	R. Hussain	Guest Worker, LPD, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Parasitic Diseases

SECTION

INSTITUTE AND LOCATION

NIAID, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goal of this project is to characterize the host immunological responses to infection with G. lamblia. These responses may modulate the clinical course demonstrated by various subsets of patients with giardiasis. We are therefore examining giardiasis patients for the presence of anti-G. lamblia antibodies in serum, salivary duct secretious and intestinal fluid by an enzyme-linked immunosorbent assay. IgG anti-G. lamblia antibodies have been demonstrated in serum. The immunoreactive antigens to which these antibodies are directed are being characterized by a combination of SDS gel electrophoresis and Staph A radioimmunoassay. To examine the cellular immune responses in giardiasis patients, we are studying in vitro lymphocyte transformation in response to G. lamblia trophozoite antigen. The effector mechanism of cellular interaction with G. lamblia is being investigated with spontaneous cell-mediated and antibody-dependent cytotoxicity assays. Thus, characterizing the humoral and cellular immunological responses in patients with giardiasis may elucidate how host defense mechanisms modulate clinical giardiasis.

Project Description:

There is mounting epidemiological, clinical and experimental evidence that immunological factors are important in host response to this parasite. The epidemiological evidence from Colorado suggests repeated exposure may confer partial protection against G. lamblia. The clinical observations that 80% of U.S. patients with immunoglobulin deficiencies and diarrhea have giardiasis supports a role for humoral immune factors in host response to G. lamblia. Finally, the experimental evidence that nude mice are more susceptible to Giardia muris suggests cellular immune factors may also be functional in host defense against giardiasis.

These immunological factors may contribute to the variable clinical course of subsets of patients with giardiasis. These subsets include asymptomatic carriers, acute symptomatic carriers who respond to antibiotic therapy, chronic symptomatic carriers with normal immunoglobulin levels and chronic symptomatic carriers with depressed immunoglobulin levels. The following projects have been established to investigate the role of humoral and cellular immunity in modulating host response to G. lamblia infection.

1a. Humoral Antibody Response. In completed work we demonstrated by an indirect immunofluorescence (IIF) antibody test the presence of serum anti-G. lamblia antibodies in 30 infected patients. This work has been extended using an enzyme-linked immunosorbent assay (ELISA) which employed recently isolated G. lamblia (see F. D. Gillin report) as antigen. This more objective test allowed us to confirm the presence of systemic antibody and show it to be of the IgG class. High antibody titer does not appear to be protective against chronic or recurrent giardiasis suggesting that the quantity of antibody is not protective. We therefore are looking at the specificity of the antibody.

1b. Antibody Specificity. To immunochemically characterize the immunoreactive antigens in G. lamblia to which human antibody is directed, SDS polyacrylamide gel electrophoresis and a Staph A radioimmunoassay in combination with autoradiography have been employed utilizing lactoperoxidase ^{125}I labeled G. lamblia protein. The system could be adopted to study all classes of human antibody. Antibody specificity will be compared in patients from different subsets and during various stages of disease.

2a. Cellular Immune Response. To assess the cellular immune response, we have established an in vitro lymphocyte transformation assay using sonicated G. lamblia trophozoites as antigen. Lymphocyte activation in 7 individuals has been studied and appears to correlate with current or past giardiasis.

2b. Cytotoxicity of G. lamblia. To study the mechanism of cellular interaction with G. lamblia, a cytotoxicity assay has been established in collaboration with D. B. Keister. This assay employs isolated human peripheral blood lymphocytes incubated with G. lamblia labeled with tritiated thymidine. Thus far we have demonstrated a high degree of spontaneous cell-mediated cytotoxicity of G. lamblia in uninfected individuals and less effector cell cytotoxicity in a patient with chronic giardiasis. The majority of the effector cell activity appears to be in the monocyte-macrophage fraction. In preliminary experiments, cytotoxicity does not appear to be antibody dependent or complement mediated.

Publications:

1. Visvesvara, G.S., Smith, P.D., Healy, G.R. and Brown, W.R.: Serum antibodies to Giardia lamblia demonstrated by immunofluorescence. Annals of Internal Medicine, in press.

LABORATORY OF STREPTOCOCCAL DIseases
1980 Annual Report
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PHS-NIH
Summary Statement
Laboratory of Streptococcal Diseases
October 1, 1979 to September 30, 1980

Research Progress

Bacterial Lipoteichoic Acid

Prior studies showed that lipoteichoic acid (LTA: a glycerol phosphate polymer) is associated almost exclusively with the mesosomes (intracellular membranous organelles) of *Staphylococcus aureus*, and that this is the major chemical difference between mesosomes and cytoplasmic membrane. Investigations of other Gram-positive bacteria have shown somewhat different distributions of their LTAs, and questions arising concern the precise nature of staphylococcal LTA, the sensitivity of methods for its detection in cellular fractions and extracellular fluids, and the origin of mesosomes as well as the nature of their association with LTA. Data obtained by an improved method of hot phenol extraction and purification on Sepharose 6B columns showed that staphylococcal LTA contained only glycerol, phosphorus, glucose, d-alanine, and fatty acids (mostly C_{15}) in a molar ratio of 1:1:0.14:0.02:0.04. Chain length of the polyglycerol phosphate backbone was 15. LTA is a detergent-like molecule which appears to be attached to the outer surface of mesosomal vesicles; studies of its critical micellar concentration, which was 100-fold lower in the native state than in the de-alininated form and thus indicated a strong tendency to aggregate into micelles, suggest that this aggregated state may account for the observed low diffusibility of LTA from the staphylococcal cell and might also explain the formation of spherical and tubular bodies identified morphologically as mesosomal vesicles. Studies with radiolabelled amino acids indicated a parallel synthesis of the proteins of mesosomes and plasma membranes, suggesting that mesosomal protein (and LTA) may be end products of metabolism, and showing that mesosomes do not arise from membrane. High titered homogeneous anti-serum to the purified LTA was highly specific for the polyglycerol phosphate backbone to the molecule as determined by inhibition of quantitative precipitin tests by glycerol phosphate and cardiolipin, and absence of inhibition by alanine, glucose, glucosamine, galactose, maltose, or ribitol teichoic acid. Purified LTA was highly effective in minute quantities in sensitizing sheep red cells, which were then used in combination with the specific antibody to develop a sensitive (0.1 mg/ml) passive hemagglutination inhibition test. The test confirmed that mesosomes contain most (65%) of total cellular LTA, whereas only 9% was found in membranes, 12% in periplasm, 6% in cytoplasm, and miniscule amounts (0.2 ml/liter) in culture fluid. These data supply a firm basis for determining the role of the mesosome in the physiology of the staphylococcus. (Huff; Theodore)

Streptococcal Bacteriophages

Phages commonly found in streptococci, and capable in many instances of transduction and lysogenic conversion, have not been adequately characterized physico-chemically. Study of 9 phages, both temperate and virulent, from 5 streptococcal groups (A, C, E, G, H) showed that 7 are typically long-tailed polyhedrons but 2 (both virulent, from Groups C and H) have short tails with complex accessory fibers. All possess linear double-stranded DNA genomes, most of which examined to date approximate ±24 Mdal in size and have both circularly permuted and terminally redundant nucleotide sequences. One virulent short-tailed phage (ø39) of Group H streptococci has a genome of half this size and lacks permuted (and possibly redundant) sequences. All DNAs are being tested for genome size, terminal redundancy (by Exonuclease III treatment), circular permutation (by denaturation-renaturation), and homologies (by comparison of restriction endonculease-generated fragments); and capsid proteins are being compared by gels. Other studies showed that 8 Group A strains producing erythrogenic toxin B (ET-B) as well as ET-A and ET-C carried temperate phages that lysogenized other strains but failed to induce ET-B production: this finding strengthens the hypothesis, resulting from prior discovery of non-lysogenic strains producing solely ET-B, that the genetic coding for this toxin is not on a prophage genome that induces a lysogenic conversion--in contrast to the production of ET-A and ET-C. Additional investigations developed, and showed the feasibility of, a method for selection of transductants to a type-specific streptococcal M-protein by use of plate-adherent immunoglobulin-binding Protein A to segregate transductants first exposed to M-specific antiserum, for subsequent agar overlay, incubation, and colony counting. (Moynet; Colon-Whitt; Popkin; Cole)

Spiroplasma Viruses and Plasmids

Prior studies established that spiroplasmas (motile, helical mycoplasmas) carry both viruses and cryptic plasmids, and investigations of their distribution were continued. Accumulated data indicate that 90% of 67 strains carry one or more of the 3 known viruses (as detected by electron microscopy), and viruses SpVl, SpV2, and SpV3 occur in 57%, 19%, and 64% of strains, respectively. One virus per strain was found in 49%, and combinations of 2 or 3 in 40%. Plaquing on presumptive in dicator lawns showed a virus in 96% of a smaller series of strains, but adjunct procedures are needed to identify the virus and plaquing efficiency on a random series of indicators (total positive/total possible) was only 28%--indicating the need for sensitive and virus-specific indicators. Both electron microscopy and gels were used to distinguish 38 sizes of plasmids in 12 of 14 strains: 12 plasmids, at least one of which was found in each strain, were shared with at least on other strain; and each strain carried from 1 to 8 plasmids. The possibility that the distinctive colonial phenotype of a non-helical mutant strain is related to loss of plasmid(s) from the parental strain (which carries 6 plasmids in contrast to one in the mutant) is being explored by use of "curing" agents, but is as yet unsuccessful. A search for appropriate phenotypic difference, in order to test possible plasmid functions is in progress. (Ranhand, Mitchell, Popkin, Cole)

Plasmid Transformation of *Streptococcus sanguis*

Three plasmids (ERL1, β, and pBD15) known to confer resistance to erythromycin (E) and linocomycin (L) in their respective originating strains of *Streptococcus pyogenes*, *Streptococcus sanguis*, and *Bacillus subtilis*, were used to transform E-L resistance to 3 strains (Wicky, WE4, Challis) of *S. sanguis*. All 3 recipients were transformed by ERL1 and β, but pBD15 was effective only in Wicky and WE4. Decreasing order of transformation efficiency was ERL1→β→pB15. The plasmid DNAs recovered from the recipient *S. sanguis* strains produced higher transformation frequencies in the same recipients than did the original "heterologous" plasmids, and their molecular weights were greater than, or equal to, those of the input plasmids: this result requires exploration, which is in progress, of intracellular changes in introduced plasmid DNAs. The most effective plasmids (ERL1 and β) transformed *S. sanguis* at greater frequencies than did homologous chromosomal DNA from a plasmid-less E-resistant strain (WE4)-suggesting different mechanisms of E resistance. In contrast to E-L plasmid-borne markers, those from *B. subtilis* or *Staphylococcus aureus* specifying resistance to chloramphenicol, tetracycline, or streptomycin, did not transform *S. sanguis*. Studies of plasmid interactions with streptococci are continuing. (Ranhand)

Streptolysin S

The major location of intracellular precursor to the Group A toxin, streptolysin S (SLS), was confirmed as the membrane fractions. The minor cytoplasmic precursor was shown to be different by sedimentability in the absence of membrane fragments, poorer activation by certain detergents, and a lower molecular weight and narrower elution profile of the form activated by the newly-discovered carrier, polyguanylic-uridylic acid (pGU). Cytoplasmic precursor may be modified (as by molecular cleavage or addition of a lipid component) when it moves to membrane, and further characterization of both forms of precursor is in process to clarify structure. Studies also showed that staphylococcal or streptococcal lipoteichoic acids (LTA) are efficient activators of precursor *in vitro*, and that the ratio of LTA to SLS in the cell is similar to that found in LTA-activated SLS--suggesting that LTA may function *in vivo* as a carrier for secreted substances like SLS. Because the fatty acid moiety of LTA was found to be the binding site of SLS, and streptococci deacylate LTA prior to its secretion, active SLS in culture supernatant fluid was not expected and not found. The finding emphasized the previous suggestion of a carrier role for LTA, and all results supply the first evidence for such a role of a naturally-occurring streptococcal component in SLS transfer. New procedures of precursor preparation, coupled with use of pGU (instead of the customary RNA-core) as activator, resulted in the highest purity and specific activity of hemolysin yet obtained, and methods of further purification and peptide analysis are being tested. Preliminary studies showed that precursor impairs polymorphonuclear cell function: further investigations of this, and of the effects of LTA-SLS in animals, are in progress to determine possible roles in pathogenicity. (Calandra; Theodore; Williams, RML; Coligan, LIG; Gerhardt, Univ. of Oregon)

Administrative, Organizational, and Other Changes

The number of research projects remained stable. Dr. Alan Liss (Sr. Staff Fellow) tranferred to RML (Laboratory of Microbial Structure and Function) in October 1979. Dr. Daniel Moynet, formerly Visiting Fellow, was appointed Visiting Associate in February 1980. Mr. Clyde Tapp (Biol. Lab. Tech. Gen.) was reemployed after a period of disability and returned to duty in November 1979. Mr. James G. Cunningham (Biol. Lab. Tech., Micro.) resigned in May 1980. Cheryl Henson (Howard University and Augustine Smith (Purdue University) came on duty in May 1980 as Biological Laboratory Aids in the Summer Undergraduate Program, to gain research experience with Drs. Calandra and Huff, respectively. Due to decreasing size of the Laboratory, doubt as to its future, and current fiscal and personnel restrictions, no major changes in or additions to, the directions of research still in progress, are anticipated.

Honors and Awards

R. M. Cole

Member, Editorial Board, Infection and Immunity.

Member, Study Group on Viruses of Mycoplasmas and Spiroplasmas: Subcommittee on Bacteriophages, International Committee on Taxonomy of Viruses.

Faculty Member, 1st International Mycoplasma Techniques Course (International Organization for Mycoplasmology and University of Bordeaux), Bordeaux, France, Sept. 3-21, 1979.

Divisional Lecturer (Mycoplasmology Division) Annual Meeting American Society for Microbiology, Miami, Florida, May 11-16, 1980.

President, Lancefield (Streptococcal) Society, 1980-81.

Symposium Convener (Recent Developments in Mycoplasma Viruses) 3rd Conference International Organization for Mycoplasmology, Custer, South Dakota, Sept. 3-9, 1980. Member, Study Group on Bacterial Virulence and Pathogenesis, NIAID.

J. M. Ranhand

Chairman and Program Coordinator, 24th Wind River Conference on Genetic Exchange, Estes Park, Colorado, June 10-13, 1980.

Invited participant, 5th European Meeting on Bacterial Transformation and Transfection, Florence, Italy, Sept. 1-5, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00001-19 LSD

PERIOD COVERED
October 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Biochemical studies on staphylococcal and streptococcal membranes, cell walls, and mesosomal vesicles.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. Huff	Sci. Director	LSD, NIAID
Other:	T.S.Theodore	Res. Microbiol.	LSD, NIAID
	A. Smith	MBRP Student	LSD, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Streptococcal Diseases

SECTION

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Md. 20205

TOTAL MANYEARS: 1.25	PROFESSIONAL: 1.00	OTHER: 0.25

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The membranes of Staphylococcus aureus and most Gram-positive bacteria consist of plasma membranes and lipoteichoic acid-containing mesosomes. The purpose of this study has been to determine the functional role of plasma membranes, mesosomes and lipoteichoic acid in the growth of the bacterial cell. Experiments have been designed to determine: (1) if mesosomes are derived from plasma membranes; (2) what degradative enzymes act on lipoteichoic acid; and (3) how the presence of alanine attached to LTA affects the dissociation of LTA aggregates.

PHS-6040
(Rev. 10-76)

Project Description

A characteristic morphological feature of Gram-positive bacteria is the presence of intracellular membranous organelles known as mesosomes, as well as plasma membranes. *Staphylococcus aureus* mesosomes can be readily prepared free of plasma membranes. Such mesosomes contain 18% lipoteichoic acid (LTA). The presence of LTA is the major chemical feature which distinguishes mesosomes from plasma membranes. LTA is a polymer of glycerol phosphate to which is attached a glycolipid and D-alanine. This structure is characteristic for a detergent molecule and accounts for the aggregation of LTA into large micelles or vesicles. Lipoteichoic acid has been implicated in the cellular binding of cations; as an inhibitor of cell wall autolysins; as a carrier for polyribitol phosphate synthesis as a mediator to binding of streptococcal cells to phagocytes; as a cause of nephropathy and chronic arthritis in rabbits; and in the peridontal inflammatory response in rats. In the present study the objective was to determine: (1) The unique chemical properties of LTA; (2) The function of LTA and mesosomes in cellular metabolism; and (3) The metabolic interrelationship of mesosome and plasma membranes.

(1) Detergent properties of lipoteichoic acid (LTA)

The most distinct chemical feature of mesosomes is the presence of lipoteichoic acid. In order to understand the physiological role of mesosomes, we felt it important to understand the unique chemical properties of LTA. LTA is a detergent-like molecule. Every detergent has a specific Critical Micellar Concentration (CMC) below which only monomer exists and above which aggregates or micelles form. These micelles are in equilibrium with monomer at the CMC. We have attempted to determine the CMC for native and dealaninated LTA by repeated chromatography of very high specific activity H^3-glycerol-labeled LTA (from *S. aureus* strain Lafferty) on Sepharose 6B. Dealaninated LTA has a CMC of 0.001%. As yet we have not been able to measure the CMC for native LTA but can estimate that it is less than 1 x 10^{-5}%. This low CMC is probably due to the association of the multiple positive (amino) and negative (phosphate) groups on the hydrophilic portion of the molecule. Thus, when the positive amino groups are removed from LTA (by removal of alanine), the micelles tend to dissociate more readily as shown by the increased CMC. At concentrations others have used to test for polyribitol phosphate polymerase, the LTA without D-alanine would be completely monomeric whereas the native LTA would be mostly aggregated. The D-alanine inhibition was considered as a simple steric effect but may instead reflect the degree of aggregation or rate of dissociation of native LTA. Lipoteichoic acid appears to be attached to the exterior surface of the mesosome. The unusually strong tendency to aggregate (low CMC) may: (1) prevent LTA from diffusing out of the cell (as observed when whole exponential cells are extracted with aqueous phenol), and (2) may account for the observed formation of vesicles and membranous tubules which are identified morphologically as mesosomes.

Since 18% of the dry weight of mesosomes is LTA, we were interested in how this would affect their solubilization by detergents. Triton X-100 at 0.02% completely dissolved mesosomes but did not affect plasma membranes. Membranes required 0.2% Zwittergent 314 for solubilization. It would appear that the LTA is mesosomes acts synergistically with the added Triton X-100 to dissolve mesosomes. As yet we do not know if LTA will dissolve plasma membranes.

(2) Biosynthetic relationship of mesosomes and plasma membranes

Although mesosomes differ from plasma membranes by the presence of LTA, similarities in their proteins (in S. aureus) by polyacrylamide gel electrophoresis were previously shown. Thus, mesosomes could be considered to be plasma membranes with attached LTA. If mesosomes are derived from plasma membranes, there should be a lag in the incorporation of radioactive amino acids into mesosomes as compared to plasma membranes. We compared constant and 2 min pulsed incorporation of S^{35}-methionine into mesosomal and plasma membrane protein. In these experiments H^3-(methyl) methionine was present for many generations and served as a measure of total methionine. The S^{35}/H^3 ratio in in protein represented a S^{35} specific activity for methionine. The ratio of the S^{35} specific activity in mesosomes to the S^{35} specific activity in plasma membranes was 0.74 when both S^{35} and H^3-(methyl) methionine were present during growth for a number of generations. Since the only other sulfur containing amino acid which could reduce the S^{35} specific activity was cysteine, it follows that the mesosomes have an elevated cysteine content. When S^{35} methionine was pulsed into cells for 2 min, the mesosome to membrane ratio was unchanged (0.74). Thus, a parallel synthesis of mesosomal and plasma membrane protein must occur. This parallel synthesis is consistent with the known constant number of mesosomes and constant LTA level during growth. These results suggest that mesosomal protein and LTA may be end products of metabolism. We plan to identify the protein(s) in mesosomes with elevated cysteine. Also we are planning a comparison of mesosomal and plasma membrane proteins using isoelectric focusing and two-dimensional gel electrophoresis.

Planned and ongoing work includes: (1) Further characterization of the protein differences between mesosomes and membranes using isoelectric focusing and two-dimensional gel electrophoresis; (2) Further characterization of the aggregation parameters of

LTA and dealaninated LTA so that non-enzymatic shifts can be distinguished from the enzymatic changes for which we are looking; (3) Determination if LTA is a cellular end product or if there is LTA turnover; and (4) Testing for specific LTA and cell wall synthetic enzymes in mesosomes and plasma membranes to determine mesosome function.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00002-15 LSD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Studies on the nutrition, physiology and ultrastructure of Staphylococcus aureus.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. S. Theodore	Res. Microbiol.	LSD, NIAID
Other:	C. G. Calandra	Med. Officer	LSD, NIAID

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Streptococcal Diseases

SECTION

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	1.0	2.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The most distinct features of gram positive bacteria are the presence of lipoteichoic acid and a membrane system consisting of a plasma membrane and mesosomes. The purpose of this project is to relate membrane structure with function using Staphylococcus aureus as the model test system. The major emphasis is to define the function (or functions) of mesosomes which contain most of the lipoteichoic acid in the cell. To support the chemical localization of lipoteichoic acid in mesosomes by immunochemical methods, antiserum to lipoteichoic acid was prepared.The activity and specificity of this antiserum to lipoteichoic acid was characterized by quantitative precipitin, inhibition, and immunoelectrophoretic studies while the localization of this polymer in mesosomes was ascertained by passive hemagglutination inhibition tests. Also, lipoteichoic acid was shown for the first time to be an activator of Streptolysin S produced by Group A streptococci.

Project Description:

Morphologically, the most distinct feature of Gram positive bacteria are the mesosomes which arise from the membranous septum and precede crosswall formation. The most distinctive chemical feature of isolated mesosomes is the presence therein of almost all of the cell's lipoteichoic acid (LTA: a glycerol phosphate polymer containing a phosphatidyl glycolipid). LTA interacts strongly with divalent cations, and acts as a regulator of autolytic activity. Also, it is immunologically active and binds to erythrocytes and other mammalian cells. Using chemical, enzymatic and serological methods, as well as subcellular fraction techniques, the overall objective of this study was to ascertain whether mesosomes are functional organelles.

(1) Antigenicity of staphylococcal LTA

The LTA used to prepare antisera was isolated from Braun disrupted cells of Staphylococcus aureus by chloroform/methanol-hot aqueous phenol treatment and purified by gel chromatgraphy on Sepharose 6b. This LTA, approximately 1.5% of the dry weight of the cell, was devoid of protein and nucleic acid (less than 1% each) and contained only glycerol, phosphorus, glucose, alanine, and fatty acids (predominantly C_{15}) in a molar ratio of 1:1:.14:.02:0.04. The chain length of the polyglycerol phosphate portion of this polymer as calculated from glycerol, phosphorous and glucose data was 15. Purity of this preparation was verified by SDS continuous and discontinuous polyacrylamide gel analyses. Electrophoresis of 500 mg of purified LTA revealed no protein bands and only one band which stained positively with Schiff-periodate reagent (carbohydrate stain).

Antibody prepared in rabbits with hot phenol LTA coupled to methylated antiserum albumin had a titer of 2.9 mg protein/ml as measured by quantitative precipitin. Inhibition studies of the quantitative precipitin reaction showed that alanine, glucose, galactose, maltose, glucosamine, and ribitol teichoic acid did not inhibit the precipitin reaction; whereas glycerol phosphate inhibited 30% and deacylated LTA (removal of fatty acids) or cardiolipin inhibited almost completely (90%). These data show unequivocally that the antibody is specific for the polyglycerol phosphate backbone of the LTA molecule. Immunoelectrophoretic analyses of LTAs isolated by hot and cold phenol and hot water extractions of disrupted or intact cells, deacylated LTA and isolated fractions of mesosome, membranes, periplasm, cytoplasm, and culture fluids, yielded only one precipitin band--further establishing the homogeneity of the antiserum.

The LTA prepared by the aforementioned procedure was of such purity that only 0.5 ug was needed to sensitize 10 ml of a 1% sheep erythrocyte suspension. In contrast, 25 ug of a lower molecular weight (peak II material) or deacylated LTA was needed for similar sensitization. Using this sensitization procedure, it was possible to detect by passive hemagglutination inhibition as little as 0.1 mg LTA/ml in any of the extracts prepared; including LTA secreted into the culture medium (0.4 mg/liter culture fluid, stationary phase cells), LTA of a S. aureus L form (0.2 mg/liter culture), and LTAs extracted from different serological groups of streptococci. When subcellular fractions of S. aureus were prepared by protoplasting, extracted only with hot phenol without further purification, and assayed by passive hemagglutination inhibition, mesosomes contained 65% of the LTA while membranes periplasm, and cytoplasm had 9, 12, and 6%, respectively.

The mounting chemical and serological evidence strongly suggests that LTA is localized predominantly in the mesosmes of S. aureus, if not all other gram positive bacteria. The question should be settled finally by immunoelectron microscopic localization of LTA in thin sections of intact cells, using the now-available specific antiserum. Since mesosomes are most evident at the time of cell division, arise from the membranous septum, precede crosswall formation, and contain LTA, it is probable that they are active in membrane, wall and LTA biosynthesis.

Proposed course of project will be immunoelectron microscopic localization of LTA in intact cells, biosynthesis of LTA and its function as it relates to the mesosome, and investigation of possible role of mesosomes in penicillin binding (since earlier studies showed that penicillinase activity is localized in the mesosomal portion of the membrane).

(2) Activation of Streptolysin S by LTA

Group A streptococci produce an oxygen stable extracellular toxin (SLS) which attacks not only erythrocytes, but also other mammalian cells. To be active, it requires a carrier molecule such as yeast RNA-core or nonionic detergent--neither of which is a native component of streptococci. Because of its cell membrane binding affinity and its possible role in the pathogenesis of streptococcal infections, we tested whether LTA could activate SLS. Staphylococcal LTA, as well as LTAs from different streptococci, activated SLS to the same extent as yeast RNA-core. SLS activity could be detected on neutral polyacrylamide gels and migrated with the LTA band; also this LTA-SLS complex could be purified on

Sepharose 6b columns and eluted in the region of LTA. This is the first time a native component (LTA) of streptococci has been shown to act as a carrier molecule for SLS and may have widespread implications with respect to its involvement in pathogenicity. LTA not only activates SLS but also may act as a carrier molecule to deliver this toxin to host tissues. Present studies are directed to further characterization of the LTA-SLS complex and possible use of this complex to purify SLS. (In collaboration with G. B. Calandra)

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00004-20 LSD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Electron Microscopy of Bacteria in Relating Structure and Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. M. Cole	Chief	LSD, NIAID
Other:	T. J. Popkin	Chemist	LSD, NIAID

COOPERATING UNITS (if any)
See following page for cooperating units.

LAB/BRANCH
Laboratory of Streptococcal Diseases

SECTION

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	0.3	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project provides support, through preparative techniques (negative staining, thin sectioning) and subsequent examination, for Laboratory and Collaborative microbiological studies requiring transmission electron microscopy. Objects of ultrastructural study include mycoplasmas and their viruses; streptococci and their bacteriophages; staphylococci; bacilli; gram-negative bacteria; fungi; and various cell fractions thereof, examined by means of cytochemistry and immunoelectron microscopy.

Cooperating Units

Laboratory of Clinical Investigations, NIAID, Clinical Mycology Section (K. J. Kwon-Chung)

Division of Infectious Diseases, Medical College of Virginia, Richmond (G. L. Archer)

Pediatric Infectious Disease Division, University of Colorado Medical Center, Denver (B. A. Lauer)

Laboratory of Infectious Diseases, NIAID, Mycoplasma Section (J. G. Tully)

Group I.N.S.E.R.M., U. 65 Montpellier-Nimes, Faculte de Medicine, Nimes, France (J. Schmitt-Slomska)

Agriculture Research Service, U.S.D.A., Beltsville, Md. (R. F. Whitcomb; D. Stiller)

Project Description

Objectives are to provide, as required, electron microscopy facilities in support of other projects in the Laboratory; and to conduct such original and in depth studies of the ultrastructure of microorganisms as may be engendered by collaborative beginnings or by independent approach to problems.

1. New strains of spiroplasmas examined for viruses (SpV) included 2 *S. citri* (105; SpV1+, SpV2+: ASP-1; SpV1+, SpV3+), 1 corn stunt (Mississippi; no virus), 4 flower (PPS1, F1, F2; no virus: L89; SpV3+), 1 bee (B13; SpV3+). The strains originated in the United States, Morocco, or France. Evidence to date indicates that 90% of 67 spiroplasma strains carry one or more of the 3 known viruses, unrelated to the original host or geographic origin of the spiroplasma.

2. There is considerable international interest, reflected in several recent publications, in the nature of internal filaments of spiroplasmas. Others have purified them and shown that they represent a protein of 55,000 daltons MW, but the site(s) and nature of their attachment to membrane in order to supply motility (if they are indeed contractile protein) remains obscure. Our attempts to localize them *in situ* by brief and minimal detergent treatment, followed by rapid fixation, embedment, sectioning, and electron microscopy, were unsuccessful. The procedures will be repeated with high dilutions of digitonin, which was recently reported effective in visualizing the cytoskeleton of mammalian cells.

3. Other mycoplasmas examined included strains of M. pneumoniae (A. Liss; J. G. Tully), Acholeplasma laidlawii and S. sp. 72-043 (J. G. Tully), and avian strains CKK and Iowa 695 (J. G. Tully). The latter were found to possess a well-defined outer layer of undefined material surmounting the membrane--a feature not noted in published descriptions of the species. The non-helical morphology of the S. citri, spiroplasma mutant, ASP-1, was affirmed by examination of cloned cultures.

4. The progress of protoplast formation in Group B streptococci treated with crude and purified preparations of mutanolysin (a new cell-wall, lytic enzyme) was examined in thin sections. (See also Z01 AI 00181-02; Calandra) Purified enzyme produced good protoplasts useful for physiologic studies, which was not possible with other known murein hydrolases: crude enzyme resulted in optical density increases that were found to be due to coagulation of cytoplasm induced, it is assumed, by other enzymes and impurities in the preparation.

5. For comparisons of known strains with recent isolates, 6 strains of staphylococci prepared both in Richmond and in our lab, were sectioned and examined for Dr. Archer (Medical College of Virginia). No unusual or unexpected differences were found.

6. Owing to interest engendered from a previously published report on an erythrocyte-associated bacterium in human infection (See Publications), we received some other examples of RBC-associated prokaryotes. Examination of thin sections of blood from a cow infected with Eperythrozoon wenyonii clearly demonstrated that this organism, like other species of the genus occurred both free and erythrocyte-associated and was an ovoid to rod-shaped prokaryote enclosed by a single limiting membrane--in which latter respect it resembles mycoplasmas more than the other members of the Anaplasmataceae. Renewed attempts at its culture, with new media, have so far been unsuccessful (Whitcomb, Stiller; U.S.D.A.). Samples of a febrile patient's blood, in which a red-cell associated bacterium had been presumably seen, were processed and examined in section, but no microorganisms have yet been found (B. Lauer, Univ. Colorado Medical Center). Because of resemblance of our original RBC-associated bacterium to published electron micrographs of the bacterium associated with Whipple's disease, we have been seeking specimens of the latter. One biopsy was furnished by Dr. Archer (Medical College of Virginia), but unfortunately (probably because of some treatment followed by remission) failed to reveal any bacteria in numerous sections examined. The problem of uncultured prokaryotes in blood or other tissues, in recognized diseases, is of interest; and more specimens will be examined as they become available.

7. Examples of parent bacteria and a stable L form (the first known) of Brucella suis were received from France (Schmitt-Slomska, Faculte de Medecine, Nimes) and examined by section. Parental forms were typical Gram-negative small rods. Colonies of the L form showed the expected protoplast-like bodies of various sizes, but were unique in the replacement of the cytoplasm of many by masses of small vesicles and tubules; and by the surrounding presence of masses and whorls of membranes of atypical dimensions. Brucella L forms are rare, and their ultrastructure has been inadequately described: examination of other strains may be required. Samples of staphylococcal L forms, also received, showed ultrastructure consistent with previous descriptions.

8. Comparison of the morphologies of 9 bacteriophages, both virulent and temperate, from several different groups of streptococci, revealed that all but 2 were long-tailed polyhedrons as usually seen in streptococci. Phage Cl of Group C streptococci, as is known, was a short-tailed polyhedron with accessory structures--as was a new phage, ø39, of Group H streptococci. Preparations of DNAs from each phage, prepared directly and after denaturation-renaturation or exonuclease III treatment and reassociation, were made on protein films by both aqueous and formamide techniques. All have been photographed for comparisons of genome, size, circular permutations, or terminal redundancies, but analysis is not complete. The DNAs will also be compared by restriction endonuclease analysis, and the phages by survey of capsid proteins, in order to enlarge the currently miniscule base of information on characterization of streptococcal phages (See also Projects Nos. Z01 AI 00005-15 and 00006-09, LSD).

9. Considerable time and effort were devoted to preparation and examination of protein film spreads of plasmid DNAs (See Project No. Z01 AI 00006-09).

10. Wild-type, virulent cells of Cryptococcus neoformans grow at 37 C and produce a melanin-like pigment on media containing L-DOPA. Irradiation-induced mutants were grossly melanin-less, did not grow at 37 C, had decreased levels of phenol exidase, and were relatively avirulent for mice (Kwon-Chung, Laboratory of Clinical Investigations, NIAID). Samples of both types of cells grown with or without DOPA, and fixed by 4 different methods, were examined in thin section with and without staining. Unstained sections verified the presence of pigment in the walls of L-DOPA-grown wild-type cells, and its absence in mutant cells. Results have been reported (Annual Meeting ASM, Miami, May 1980, Abstract F16).

Publications

Archer, G. L., Coleman, P. H., Cole, R. M., Duma, R. J., and Johnston, C. L., Jr.: Human infection with an unknown erythrocyte-associated bacterium. N. E. J. Med. 301: 897-900, 1979.

Cole, R. M., and Popkin, T. J.: Electron microscopy. In Manual of Methods for General Bacteriology, Chap. 1, Morphology. American Society for Microbiology. (In press)

Calandra, G. B., and Cole, R. M.: Lysis and protoplast formation of Group B streptococci by mutanolysin. Infect. Immun. (In press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00005-15 LSD

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies on bacteriophages and genetics of streptococci

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. Colon-Whitt	Microbiol.	LSD, NIAID
	R. M. Cole	Chief	LSD, NIAID
Other:	D. Moynet	Visit. Assoc.	LSD, NIAID

COOPERATING UNITS (if any)

Laboratory of Parasitic Diseases, NIAID (T. Mercado)

LAB/BRANCH

Laboratory of Streptococcal Diseases

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.1	1.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of this project is clarification of the nature of bacteriophages and of mechanisms of genetic transfer (lysogenic conversion, transduction, transformation) among streptococci, and determination of their effects on pathogenicity and immunogenicity. In current efforts to transduce type-specific M-proteins, we are evaluating the efficiency of protein A-coated plates to bind cells exposed to specific antibody, for subsequent incubation and scoring of transductant colonies. Continuing studies of erythrogenic toxin failed to incriminate lysogeny in production of ET-B, in contrast to ET-A and ET-C. Other studies showed that the extracellular factor of Pseudomonas fluorescens lyses Trypanosoma cruzi in vivo as well as in vitro, causing a drop in parasitemia and decreased tissue damage. All above areas of investigation are continuing.

Project Description

(1) Transduction of antigenic markers in Group A streptococci

Previously, we reported intro-group and inter-group transduction of the Sm^+ marker (streptomycin resistance) in streptococci. Current efforts are directed to transduction of antigenic markers (primarily M protein) in Group A streptococci. The importance of this type-specific cell-surface protein in virulence and pathogenicity makes these studies highly desirable. However, selection of antigenic transductants has proved a major difficulty. A recent literature report utilized the bactericidal capacity of normal human blood for M^- streptococci to select surviving M^+ transductants. However, this method restricts recipients to M^- strains (which are rare), is unwieldy, and does not readily permit plate scoring to determine transduction efficiency. A more effective method could be the binding of specific antibody-coated transductants to plastic petri dishes coated with immunoglobulin-binding staphylococcal protein A, followed by saline washing to eliminate unbound non-transductants, overlaying with agar medium, and incubation to permit colony formation and scoring. In preliminary tests of feasibility, mixed cultures of M-6 Sm^R and M-12 strains, grown in the presence of anti-M-6 serum, were treated in this fashion, using agar overlays with and without streptomycin. In both, from 60-70% of input M-6 Sm^R colony-forming units were recovered, although about 5% of M-12 cfu were also found only on the plate without streptomycin. We are refining this technique, and in the near future will apply it to selection of M^+ transductants.

(2) Erythrogenic toxin

Erythrogenic toxins (ET, or streptococcal pyrogenic exotoxins SPE), are extracellular proteins produced by a number of group A streptococci. Four distinctive antigenic types (A,B,C, and D) have been described and lysogeny has been considered essential for their production. In previous reports we described a rare non-lysogenic strain (64 x 402) that produced ET-B, but upon lysogenization or vegetative infection by a phage isolated from group A strain C203U (producing ET-A and C) synthesized ET-C but not A, in addition to ET-B. These results suggested that (1) strain 64 x 402 may be carrying a defective, and therefore undected phage, or (2) lysogeny may not be essential for the production of ET-B toxin. However, attempts to isolate a defective phage by recombination with a virulent mutant phage were unsuccessful. Eight $ET\text{-}B^-$ strains lysogenized by phages from $ET\text{-}B^+$ strains failed to synthesize ET-B toxin. Similar results were reported recently in the literature. A supposedly non-lysogenic strain $T25_3$) produced ET-A and B and acquired ET-C toxin by lysogenic conversion. However, we found this strain to be lysogenic. We "cured" it of phage by U.V. irradiation and isolation of the surviving colonies, followed by 5 consecutive passages on blood agar plates. Currently we are testing the "cured" and the "parent" strains for ET production, using antisera and methods previously described. Determination of location of genetic coding for ET-B toxin will require study of a large number of ET-B$^+$ strains. Although previously rare (ET-A$^+$ predominated), these as well as ET-C$^+$ strains are now commonly isolated (according to investigator colleagues elsewhere) and will be made available for determination of lysogenicity and of the role, if any, of phage in production of ET-B toxin.

(3) Lysis of *T. cruzi* by *Pseudomonas fluorescens*

In collaboration with Dr. T. Mercado (LPD) we have been studying the effect of *P. fluorescens* in *T. cruzi*. Last year we reported the observation that this gram-negative, motile rod appeared to have an affinity for the kinetoplast-flagella site of *T. cruzi*, where it attached, eventually causing morphological changes of parasite. Filtered and concentrated (10X) supernatants of the bacterial cultures caused the same effect, with complete lysis within 10 min after exposure. This anti-trypanosome factor (ATF) appeared to be extracellular in nature, and its main activity found during the late logarithmic phase of growth. This year we continued our efforts to characterize this factor and assess its activity *in vitro* and *in vivo*.

Chemical tests showed ATF is resistant to heat (56 C for 1 hr), freezing (-20 C), and trypsin (25 ug/ml), but not to pronase (20 ug/ml). Gel filtrations and high performance liquid chromatography disclosed at least one active fraction that appears to be a small peptide of a molecular weight between 8,000 to 12,000 daltons. Work is in progress to further purify the factor. Biological assays of the factor showed that as little as 24 ug/ml protein caused instant paralysis and lysis (within 24 hr) of the parasite.

ATF lytic activity against other hemoflagellates was also observed (e.g., *Leishmania brasiliensis* *T. equiperdum*, and two myotropic strains of *T. cruzi*).

Other bacterial species (e.g., *P. aeruginosa*, *B. subtilis*, and *E. coli*) were also tested for production of ATF. Only *P. aeruginosa* supernatants showed any lytic effect on *T. cruzi*.

Preliminary observations indicate that ATF is also active *in vivo*. Leukocytosis with a marked drop in parasitemia was observed following 2 or 3 intramuscular injections in heavily infected mice. Microscopic examination of liver, spleen, heart, and skeletal muscle revealed considerably less damage in ATF-treated infected mice when compared to untreated infected controls. These findings are currently udner more extensive investigation.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00006-09 LSD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Competence development and genetic exchange mechanisms in streptococci.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Jon M. Ranhand	Sr. Scientist	LSD, NIAID
Other:	Daniel Moynet	Visit. Assoc.	LSD, NIAID
	W. O. Mitchell	Biologist	LSD, NIAID
	T. J. Popkin	Chemist	LSD, NIAID
	R. M. Cole	Chief	LSD, NIAID

COOPERATING UNITS (if any)
Macromolecular Biology Section, LBV (F. DeFilippes)
Z01 AI 00126-07 LBV

LAB/BRANCH
Laboratory of Streptococcal Diseases

SECTION

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.75	2.00	0.75

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

I examined genetic exchange (transformation) between heterologous and "homologous" plasmid DNAs and the group H streptococci, strains WE4, Wicky, and Challis. The three strains were transformed to erythromycin or lincomycin resistance when treated with plasmid DNAs derived from Streptococcus pyogenes strain SM 60, S. faecalis strain DS5, and Bacillus subtilis strain BD431. These strains harbor ERL1, pAM beta 1, and pBD15, respectively. The transformation order for heterologous plasmids was: ERL1 was greater than pAM beta 1 which was greater than pBD15. Plasmids isolated from transformed clones (homologous plasmids) transformed the wild-type parents at higher frequencies. An explanation for this is that the surviving plasmids are enzymatically modified.

PHS-6040
(Rev. 10-76)

Project Description:

Part I

Last year, I reported on the reactions between competent *Streptococcus sanguis* cells, strain WE4, and various DNA molecules. The reactions studied were: total DNA binding, DNAse I-resistant DNA binding, and the degradation of DNA. For most DNAs, the last reaction resulted in destruction (production of acid-soluble fragments) of about 40 to 70% of the molecules added. Plasmid DNA (pMB9) derived from *Escherichia coli* cells behaved similarly.

A report in the literature (LeBlanc and Hassel, J. Bacteriol. 128: 347-355, 1976) showed that *S. sanguis* Challis cells could be transformed with plasmid DNA derived from *S. faecalis* cells, strain DS5. The genetic marker transformed was resistance to erythromycin. Knowing that a great amount of plasmid DNA was destroyed during the reaction between competent cells and DNA, I was interested to see if the reported result could be repeated and, in addition, I wanted to see if all competent *S. sanguis* cells treated plasmid DNA the same way. LeBlanc and Hassel reported a 1.5 million decrease in the recoverable molecular mass.

A major part of this work involved defining conditions that allowed the isolation of plasmid DNAs from streptococci. I combined a few published methods and got reasonable results although there are still areas of the protocol that need refining and further modifications. When these are worked out I will then include them in a report.

Plasmid DNAs were purified from the following. ERL1 from *S. pyogenes*, strain SM 60; pAM alpha 1, pAM beta 1, and pAM gamma 1 from *S. faecalis*, strain DS5; pBD15 from *B. subtilis*, strain BD431; pSA0501 from *Staphylococcus aureus*, strain RN 2443; pSA2100 from *St. aureus*, strain RN 2444. ERL1, pAM beta 1, and pBD 15 confer resistance to erythromycin and lincomycin; pSA0501 confers resistance to streptomycin; PSA2100 confers resistance to streptomycin and chloramphenicol; pAM alpha 1 confers resistance to tetracycline; pAM gamma 1 codes for a hemolysin. The staphylococcal plasmids did not transform any of the *S. sanguis* cells used. Neither did pAM alpha 1 nor pAM gamma 1. Of the remaining plasmids, ERL1 transformed all *S. sanguis* cells the best; frequencies ranged from 0.004 to 0.02 (per colony forming unit). pAM beta 1 was next best with frequencies ranging from 1 per million to 1 per 5million; pBD15 was least active with frequencies ranging from 1 per 60 million to 1 per 700 million. Challis cells did not transform with pBD15 DNA. The original observation by Leblanc and Hassel was repeated and extended. It should be stated here that repeating the above work was not consistent for unknown reasons.

Clones from transformed cells were picked and regrown in erythromycin and lincomycin. Plasmid DNAs were purified and assayed on agarose gels using standard protocols. The following was observed. WE4, Wicky, and Challis cells treated pAM beta 1 differently. In WE4 cells three plasmid DNA bands were visualized having molecular masses of 12.5, 18.1, and 28.8 megadaltons (Mdal). The cells were sensitive to tetracycline and did not produce beta hemolysis on human or horse blood. Wicky cells contained four plasmids with molecular masses of 6.5, 7.7, 9.2, and 14.9 Mdal. These cells were also sensitive to tetracycline and did not produce beta hemolysis on human or horse blood. Some Challis cultures, although resistant to erythromycin and lincomycin (less than a 1 per 400 million chance occurrence), did not contain any plasmid DNAs. When tested, the "chromosomal" DNA band transformed WE4, Wicky, and Challis cells to erythromycin or lincomycin resistance. It is presently assumed that either all or part of pAM beta 1 integrated with the chromosome. Future work should determine this. It is concluded from the above that pAM beta 1 can behave differently in different hosts (clones) of the same strain.

When plasmids from S. sanguis clones that were transformed with ERL1 were examined they had a molecular mass of 23.5 Mdal. The plasmid as isolated from S pyogenes SM60 had a molecular mass of 28.3 Mdal. Therefore, the plasmid suffered a 4.8 Mdal deletion. This deletion may be a consequence of the uptake process. It is difficult to believe that DNA can pass into a competent cell without some destruction.

The plasmids isolated from WE4 and Wicky cells that were transformed with pBD15 DNA from B. subtilis cells had a molecular mass of 25.3 Mdal; the original mass was 2.2-2.3 Mdal. This means that the plasmid fragments either recombined with themselves or formed concatenates upon replication.

It is concluded from the above that S. sanguis cells treat different plasmids differently.

A few years ago, we reported that S. sanguis cells that contained a lysogenizing virus could not bind DNA, and therefore, could not be transformed. Therefore, it was of interest to determine if cells containing plasmids could be made competent for transformation. Such cells did transform when treated with chromosomal DNA containing the genes that confer resistance to streptomycin.

From the data obtained, it is tentatively concluded that S. sanguis cells can modify and restrict foreign DNAs. This conclusion comes from the observation that once a foreign plasmid DNA has become successfully propagated in the cytoplasm, it can then transform other S. sanguis cells at higher frequencies ranging from 10,000 to 1 million times. There is no evidence that established plasmids are amplifiable with chloramphenicol.

Because of the interest in streptococci as cloning vehicles, I plan to continue this work and hope to elucidate the plasmid transformation process.

Part II

Spiroplasma plasmids. Prior work with W. O. Mitchell, T. J. Popkin, and R. M. Cole (See Projects Z01 AI 00007-06 LSD and Z01 AI 00004-20 LSD) was extended, confirmed, and published. Present data show that 12 of 14 spiroplasmas carry from 1 to 8 cryptic plasmids per strain. Sizing by both electron microscopy and agarose gel electrophoresis (the latter using new standards) showed a range of molecular weights between 1×10^6 and 26×10^6 daltons. Several size classes occurred in common in different spiroplasma strains. The amount of plasmid DNA per strain was as much as 12% of total cellular DNA, as determined by radioactive labelling. No such multiplicity of plasmids, with some in common among several strains, were previously known in any mycoplasmas, and their newly-recognized presence in spiroplasmas raises questions of their relation to phenotypic traits, that have yet to be answered. Preliminary studies showed that a non-helical "mutant" with a distinctive colonial morphology lacks several plasmids present in the parent strain, but trials of plasmid curing of the latter by use of intercalating agents to produce "mutant" colonies have not yet been successful. Future work will be directed to this and other approaches to determining the functions of spiroplasma plasmids, and to their detection and characterization in strains not yet examined.

Streptococcal bacteriophages. Because of the paucity of current information, Dr. Moynet (in collaboration with A. colon-Whitt: Z01 AI 00005-15; See also Z01 AI 00004-20) initiated studies to characterize several phages. Nine phages, of which 3 are virulent and 6 temperate, from streptococci of serologic groups A, C, G, E, and H, are being compared. Seven are long-tailed polyhedral phages (Type B) as are most streptococcal phages, but 2 are short-tailed with accessory fibers. DNAs from all have been prepared, and are being analyzed directly for size, after denaturation and renaturation for the presence of circular permutations, and after exonuclease III treatment for the presence of terminal repetitions of nucleotide sequences. Early results suggest smaller molecular size and absence of permutation in DNA of short-tailed phages. Other studies in progress include comparison of restriction enzyme cleavage patterns to determine regions of homology in the DNAs, and gel electrophoretic comparison of capsid proteins.

Dr. Moynet has also continued physical mapping of the genome (a 24.1 Mdal linear ds-DNA) of Group H streptococcal phage ø42.

Because of the previously shown presence of circular permutation, the map--when completed--will be circular. By the use of single and double digests with restriction endonucleases (chiefly Sal I and Pvu II), followed by ordering and sizing of resultant fragments by agarose gel electrophoresis, and of treatments with large fragment Polymerase I in the presence of [^{32}P]-thymidine triphosphate, it was shown that ø42 DNA possesses 2 Sal I sites that encompass a Pvu II site. One of these now-identified Sal I sites has been chosen arbitrarily as the origin, and construction of the map will proceed.

Hemophilus bacteriophage Physical mapping of the genome of øN3 of *H. influenzae* was partially completed in 1977-78 (E. Jablonska, Visiting Fellow: See Project No. Z01 AI 0006-7 LSD), but ambiguities existed among small fragments, in the middle of the genome, generated by restriction endonucleases EcoRl, XhoI, and Xbal. Resolution of this problem is being approached by Dr. Moynet and Dr. Jablonska (as a short-term Guest Worker) by filter hybridization of EcoRl and Xhol fragments with Xbal fragments, and is expected to lead shortly to completion of the map.

Publications

Ranhand, J. M.: DNA binding and uptake by competent *Streptococcus sanguis* Wicky cells. In Glover, S., and Butler, L. O. (eds.). Proc. of the 4th European Meeting on Transformation and Transfection. Cotswold Press, Oxford, 1979, pp. 60-72.

Ranhand, J. M.: Interactions of competent *Streptococcus sanguis* (Wicky) cells with native or denatured, homologous or heterologous deoxyribonucleic acids. J. Bacteriol. 142: 568-580, 1980.

Ranhand, J. M., Mitchell, W. O., Popkin, T. J., and Cole, R. M.: Covalently-closed circular Deoxyribonucleic acids in spiroplasmas. J. Bacteriol. (In press: Vol. 143, Sept. 1980)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00007-05 LSD

PERIOD COVERED
October 1, 1979 to September 30,1980

TITLE OF PROJECT (80 characters or less)

Viruses of Spiroplasmas and Mycoplasmas

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. M. Cole	Chief	LSD, NIAID
Other:	W. O. Mitchell	Biologist	LSD, NIAID
	T. J. Popkin	Chemist	LSD, NIAID

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Streptococcal Diseases

SECTION

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Md. 20205

TOTAL MANYEARS: 4.3	PROFESSIONAL: 1.8	OTHER: 2.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project aims to characterize the nature, distribution, and significance of viruses and plasmids carried by spiroplasmas Accumulated data show that approximately 90% of spiroplasmas, as determined by either electron microscopy or plaque formation on indicator lawns, carry 1 or more viruses: 49% carry only 1, and 40% carry either 2 or 3 simultaneously. About 86% carry also, per strain, 1 to 8 of 38 cryptic plasmids--of which 12 are shared by 1 to 3 other strains. Current studies include examination of new strains for viruses and plasmids, investigation of possible differences in viral susceptibilities among cells in spiroplasma cultures, and attempts to cure plasmid-bearing strains to alter host phenotypes.

PHS-6040
(Rev. 10-76)

Project Description

An additional 9 newly-acquired spiroplasma strains were cultured and examined for viruses (See Z01 AI 00004-20). A review of data to date indicates that, as detected by electron microscopy, 90% of 67 strains carry 1 or more of the 3 known viruses. SpV1 is carried by 57%, 19% carry SpV2 (Found in *S. citri* only), and 64% carry SpV3. A single virus per strain was carried by 49% of strains, and combinations were found in 40%: only 10% represented triple combinations, whereas 2 viruses together were found in 30% of strains. The use of cross-plaquing methods indicated differences according to both donor and recipient strains, and based on the number of plaques resulting as related to the number of possible if all combinations were successful, was only 28% efficient overall. Nevertheless, the detection of at least one plaque on at least one recipient lawn, in an unselected series of 25 strains used as both donors and recipients, showed a slightly greater efficiency (96%) than electron microscopy in detecting the presence of virus. However, this method requires additional procedures to determine the morphologic type of virus and to verify origin of the isolate. Highly sensitive and virus type-specific recipients, would be desirable, but are not yet available for use in culture screening.

Grossly overt indications of infection with spiroplasma viruses, or of spontaneous viral replication, have not been described. One possibility, explored in collaboration with R. E. Whitcomb (u.S.D.A., Beltsville) is the sudden occurrence of poor growth of a strain. One such strain (*S. citri* 105) exhibiting this phenomenon consistently produced both large and small colonies, and the latter grew poorly in broth media after a long lag. Electron microscopy of the latter revealed massive infection with SpV1, whereas large colony growth showed only small number of viral particles. The possibility is being considered that two populations of host cells, with differing susceptibilities to viral infection, may occur or develop (possibly as a result of "mutation") in a culture, and thus account for the unpredictable surges of massive virus production that we have repeatedly observed over a period of time in many regularly passaged cultures. Planned studies will involve precise cloning procedures and tests of sensitivity to virus, to test this hypothesis.

Most of the effort of personnel in this project during the past year has been devoted to investigation of the newly-discovered plasmids of spiroplasmas. (See also Projects Z01 AI 00006-09 and Z01 AI 00004-20 LSD) For verification of prior results and extension to other spiroplasmas, 22 strains were grown in 100 ml to 500 ml lots: these are stored frozen and/or with glycerol, and will be comparatively screened directly by a revised agarose gel electrophoresis method developed by Dr. Ranhand

(Z01 AI 00006-09 LSD). Results to date, by different techniques, show that 12 or 14 spiroplasmas of widely diverse origins carry a total of 38 sizes of covalently-closed circular DNAs (plasmids), of which 12 were found in from 2 to 4 different strains. From one to 8 sizes were found per strain, and each of the 12 positive strains carried at least one plasmid (some 4 or 5) found also in one or more other strains. Most strains examined were *S. citri*, and most of the 12 shared plasmids were found in *S. citri* strains: however, 3 were shared with a bee strain, and one with a corn stunt strain. Information is as yet inadequate to relate the presence of given plasmids to serologic grouping or other attributes of spiroplasmas. In attempts to establish a useful system for examining phenotypes, a non-helical spiroplasma mutant of *S. citri* (ASP-1) with a distinctive colony morphology and lacking a 39,000 MW protein, was compared with its parental strain (SP-A). The latter was found to carry 6 plasmids, whereas the mutant carries only one of these (a 4.1 Mdal plasmid). No system for plasmid transfer in spiroplasmas is known, so attempts were made to "cure" the strains of plasmids by treatment with acridine orange and ethidium bromide. To date, treated cultures of SP-A have not revealed colonies typical of ASP-1 (nor vice-versa), but the study is continuing and similar methods will be applied to other appropriate phenotypes as they become available.

Publications

Ranhand, J. M., Mitchell, W. O., Popkin, T. J., and Cole, R. M.: Covalently-closed circular deoxyribonucleic acids in spiroplasmas. J. Bacteriol. (In press: Vol. 143, Sept. 1980)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00181-02 LSD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Isolation, purification, and characterization of toxins and inflammatory factors of streptococci.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. B. Calandra	Med. Officer	LSD, NIAID
Other:	T. S. Theodore	Res. Microbiol.	LSD, NIAID
	T. J. Popkin	Chemist	LSD, NIAID
	R. M. Cole	Chief	LSD, NIAID

COOPERATING UNITS (if any) Arthritis and Rheumatology Branch, NIAMDD (R. Wilder); Lab. of Immunogenetics, NIAID (J. Coligan); Lab. of Structure and Function, RML, NIAID (J. Williams); and Univ. of Oregan Medical School, Dept. of Microbiol. (N. Gerhardt).

LAB/BRANCH
Laboratory of Streptococcal Diseases

SECTION

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.3	1.3	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this work is to characterize several toxic factors of Group A streptococci that may play a role in streptococcal pathogenesis. The study of streptolysin S (SLS) includes its purification, characterization, and determination of its mechanism of secretion from the bacterium. Since lipoteichoic acid (LTA) was discovered to be a carrier molecule for the SLS hemolysin, the relationship of the two in the bacterium and in possible pathogenic roles are being explored. The difference in binding to several different carriers such as detergents, synthetic polynucleotides, and LTA is being utilized both for purification of the toxin and to distinguish the different intracellular forms of the toxin. Group A streptococcal cell walls which can produce severe rheumatoid-like arthritis in rats are being utilized to determine the genetic differences in host response which lead to the arthritis. Macrophages and lymphocytes of responder and non-responder rats are being analyzed and compared to determine the basis of response.

PHS-6040
(Rev. 10-76)

Project Description

Streptolysin S is the most powerful membranolytic agent known. Yet the role of this hemolysin of Group A streptococci in pathogenesis is unknown because no antibody response has been detected in either man or test animal. The toxin is made as an inactive precursor intracellularly--the majority associated with the membranous fraction of the cell. It has been found in high titer in all nephritogenic strains tested. The active hemolysin is likely a very small peptide which is very cationic and has high affinity for lipids. The objective is to purify the hemolytic moiety using the different chemical characteristics of the precursor and active hemolysin, to characterize it, and to determine its potential role in streptococcal disease such as glomerulonephritis. This and other toxins and inflammatory factors from streptococci are being characterized to determine potential roles in disease production.

(1) Streptolysin S

Streptolysin S in the streptococcus is an inactive substance termed SLS precursor that can be activated when blended on a Vortex mixer with a carrier such as RNA-core. There are a number of important questions. (1) How is SLS precursor activated by the streptococcus? (2) How is SLS secreted? (3) Can an infected host activate SLS precursor? (4) Do the cellular forms of SLS have adverse effects on an infected host? To examine the first two questions, studies were done to define new carriers that could be used as probes to characterize SLS precursor and to purify SLS. Of the synthetic nucleotides examined, poly guanylic-uridylic acid (pGU) functioned almost as well as RNA-core, but, more importantly, when activated with pGU, SLS precursor from membrane and cytoplasm could be distinguished from each other. By gel filtration active hemolysin from cytoplasmic precursor had a smaller molecular weight and narrower elution profile than that from membrane precursor. (This is in contrast to the lack of differences in gel filtration of hemolysin activated with RNA-core from membrane or cytoplasmic precursor) Tween 40 or 60 was also useful as a probe to distinguish cytoplasmic precursor, which it activated very poorly, from membrane precursor which it activated better than did RNA-core. SLS precursor from cytoplasm is modified, perhaps by cleavage of the molecule or by addition of another component such as a lipid, when it moves to the membrane. Results from additional studies suggest that extracellular SLS is similar to that from the membrane. Current work is directed toward characterizing precursor from membrane and cytoplasm to determine the physical changes involved in the secretion of the toxin.

Streptococcal lipoteichoic acid (LTA) may be important for streptococcal adherence to mammalian cells, but other roles for LTA in pathogenesis have not been defined. One possibility is that LTA may function as a carrier or transport substance for substances secreted through the membrane. When SLS precursor was blended with glass beads in the presence of Group A streptococcal LTA, SLS was activated. Although LTA from staphylococcus or Group B, C, or G streptococci was also effective, this was not unexpected since antigenically and chemically they are the same. Calculations of the cellular content of both LTA and SLS precursor showed that the relative proportions were similar to those used to activate SLS in vitro. Chemically the fatty acid portion of LTA was shown to be the SLS binding site, since deacylation of LTA rendered the molecule ineffective as a carrier. Since streptococci in culture normally deacylate LTA prior to its secretion, no active SLS would be expected in the culture fluids and none is found. Work is in progress to determine if LTA and SLS interact in the streptococcal cell. This is the first evidence that a streptococcal factor can function as a carrier for SLS. It is important to examine the effect of LTA-SLS on an infected host, since previous work on the effect of SLS on host has used only RNA-core SLS, an artificial toxin probably never formed in vivo. (In collaboration with T. S. Theodore, Project No. 00002-15 LSD)

Purification procedures for SLS have involved use of pGU as a carrier for the hemolytic moiety. Using a new procedure to prepare SLS precursor, the specific activity of pGU-SLS purified only by ethanol precipitation and sephadex chromatography exceeds 7×10^6 hemolytic units per mg protein. The degree of purity using only these simple steps far exceeds that ever obtained before. The hemolysin can be further purified by isoelectric focusing and neutral gel polyacrylamide electrophoresis, but not as yet (due to loss or instability of pGU-SLS) by high pressure liquid chromatography. (In collaboration with J. Williams, RML) Since further purification depends upon peptide analysis, a collaborative effort with Dr. John Coligan has been undertaken.

Preliminary studies of partially purified SLS precursor in cell membranes showed that polymorphonuclear cell function was impaired, suggesting that the cell-bound forms of SLS (SLS precursor) may adversely effect the host. (In collaboration with Nancy Gerhardt, Univ. Oregon Med. Sch.) Studies of the effect of LTA-SLS on host (test animal) functions (renal and cardiac) are also in progress.

(2) Toxins of Groups B, C, and G streptococci

A procedure was developed to prepare protoplasts of Group B streptococci. This will make it possible to determine certain enzymatic and antigenic properties of the Group B streptococcus. A low titer cell-bound hemolysin distinct from streptolysin S has been characterized, but due to its lability and low titer, its role as a pathogenic factor is uncertain.

(3) Peptidoglycan-induced arthritis

When cell wall fragments of Group A streptococci are injected intraperitoneally into rats, they develop arthritis that look clinically and pathologically like rheumatoid arthritis. In the evaluation of over a dozen inbred rat strains, three groups of animals have been defined. One group develops extensive disease, one group is resistant to the development of arthritis, and there is an intermediate group. The response does not appear linked to any histocompatibility locus or, as yet, to any defined marker. Using matings and backcrosses between responders and nonresponders, the genetic parameters of susceptibility to arthritis are being defined. Since the response is macrophage-mediated and presumably T-cell dependent, the cellular difference between responders and nonresponders are being examined. (In collaboration with R. Wilder,NIAMDD)

Publications

Calandra, G. B., and Cole, R. M.: Lysis and protoplast formation of Group B streptococci by mutanolysin. Infec. Immun. (in press)

Calandra, G. B.: Effect of detergents on streptolysin S precursor. Infec. Immun. (in press)

Calandra, G. B., and Whitt, R. S.: Activation of streptolysin S-related cellular potential hemolysin by grinding with glass beads. J. Clin. Microbiol. (in press)

LABORATORY OF VIRAL DISEASES
1980 Annual Report
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PHS-NIH
SUMMARY REPORT

ANNUAL REPORT OF THE LABORATORY OF VIRAL DISEASES, NIAID
October 1, 1979 - September 30, 1980

Dr. Wallace P. Rowe
Chief, Laboratory of Viral Diseases

The scientific program of LVD has been focused almost exclusively on the murine C-type virus problem during the past year and the general direction of the work has expanded into a large and highly productive collaborative program with the Recombinant DNA Unit in OSD. The use of molecularly cloned genomic fragments of various C-type viruses is providing an unparalleled technique for studying the organization and structure of endogenous C-type viral genomes. This technology, coupled with the biological materials available from our long experience in this field provides a truly exceptional opportunity to biochemically characterize in detail these endogenous chromosomal genomes. Another collaborative effort has increased in scope and productivity, that is the collaboration with LMI investigators focusing on the expression of C-type viruses in cells of the immune system as measured by serologic, functional, and virologic techniques. This collaborative effort like the LVD program itself draws very heavily on mouse genetics and complements the genetic studies that we have been doing in very effective ways.

Another new direction in the LVD program is increased emphasis on the role of various murine C-type viruses in spontaneous and carcinogen induced disease in various strains of mice. Analysis of the pathogenicity of various MCF viruses in combination with various helpers is a major goal and one that may be of much importance for putting these viruses in perspective. It is now clear that pathogenicity of MCF viruses cannot be measured simply by inoculating them into suspectible suckling mice but that the helper effect of endogenous or perhaps exogenous ecotropic virus is a major determinant of their ability to cause disease. Simple models whereby the virus is created, infects and transforms, and thereby causes disease are no longer adequate to test the complex host-cell interactions that occur in mice. Also, we hope soon to initiate studies using our congenic NFS mouse strains into which various endogenous murine C-type viruses have been introduced on a common inbred mouse genetic background in order to more precisely analyze whether these viruses contribute to chemical or radiation leukemogenesis. Among the congenic mice we have strains carrying high inducibility loci, low inducibility loci, or high inducibility loci whose virus products are so highly immunogenic that the infection is almost completely suppressed; a strain in which the spread of virus is restricted by being bred into mice carrying an incompatible Fv-1 allele; and strains in which high inducibility alleles are phenotypically suppressed by the production of maternal antibody at each generation. Study of the response of these strains to standardized carcinogenic regimens should clarify some questions about mechanisms of interaction of carcinogens with endogenous viruses, for example, whether immune function, induction of virus, or heightening of cellular susceptibility to exogenous infection play any role. The role of MCF generation in each of these biological situations will also be assessed.

The exceptional opportunities of recombinant DNA technology and the variety of experimental approaches and disciplines (available in our laboratory or provided by strong collaborators) now focused on various aspects of the LVD C-type virus program create an exceptional ability to examine important biological questions. The range of biochemical, biological, immunological, genetic, and pathogenetic expertise as well as our exceptional ability to apply somatic cell genetic techniques to these questions are a remarkable combination.

Identification of a gene that specifically restricts MCF virus infection. A new MuLV resistance gene has been detected in DBA and certain other inbred mouse strains. Dominant in crosses with sensitive mice and active in vivo as well as in tissue culture, it is unlinked to Fv-1 and appears to specifically affect MCF virus infection and spread. It is therefore an important factor in analysis of the genetics of virus induced and spontaneous leukemogenesis. (Hartley; Morse, LMI)

Leukemogenic activity of MCF viruses dependent on presence of ecotropic virus. In hybrid mice segregating for endogenous AKR ecotropic MuLV expression, MCF virus-induced lymphomagenesis was found to be dependent on replication of the MCF virus, which was in turn dependent on presence of the ecotropic virus. An understanding of the mechanism of this helper effect will be crucial in evaluating the biological differences between leukemogenic and non-leukemogenic MCF viruses and in defining further this complex host-virus relationship. (Cloyd, Hartley, Rowe)

Ecotropic MuLV leukemogenesis regularly accompanied by generation of MCF viruses. MCF viruses are readily detected in preleukemic and leukemic mice of most high ecotropic virus strains. We have also found that infection of virus-negative mice with cloned leukemogenic ecotropic viruses, such as Friend, Moloney, and isolates from wild mice, regularly results in generation of MCF viruses, sometimes as early as 3 weeks after infection. MCF viruses are regularly detected in both erythroid and lymphoid leukemias. This finding provides a system with defined virological background for examining genetic and immunological factors controlling recombinant virus formation and spread. (Hartley)

Detection of germ line reinsertions of ecotropic MuLVs. Several sublines of NIH-Akv-1 mice, a congenic line carrying the Akv-1 ecotropic virus-inducing locus on chromosome 7, were found to have developed during inbreeding additional viral loci unlinked to the Akv-1 locus markers. The rare occurrence of germ line viral gene reinsertions is probably the means by which high virus mouse strains evolve and determination of the mechanism and its regulation will be of importance. (Rowe, Kozak)

Chromosomal mapping of Akv-2. The chromosome 16 association of the Akv-2 virus-inducing locus of AKR, suggested by use of somatic cell hybrids, has been confirmed by standard mendelian crosses; close linkage to the mahoganoid locus was observed. (Kozak, Rowe)

Isolation of single chromosomes carrying virus-inducing and virus-regulating loci into somatic cell hybrid lines. Clonal mouse-hamster hybrid cell lines are being selected for possession of individual MuLV-related genes involved in induction and regulation. These lines will be useful in studies of the kinetics of virus induction, genetics of restriction of different MuLV classes and of expression of MuLV antigens, and for biochemical studies of the organization of endogenous genomes. (Kozak)

Biochemical detection of ecotropic proviruses. A molecularly cloned probe specific for ecotropic MuLV has been derived and characterized. It is in use in conjunction with cleavage with several restriction enzymes for analysis of genomic DNA from various inbred mouse strains with different endogenous genome arrays and expression. (Martin, Chan, Rowe)

Identification of a unique class of ecotropic-reactive sequences in DNA of ecotropic-negative mice. Several strains of mice known to be negative for expression of ecotropic virus and to lack the complete viral genome have been found to possess an unusual class of DNA sequences which hybridize with the ecotropic-specific probe. This is an unexpected finding and further analysis is in progress. (Martin, Chan, Hoggan, Rowe)

Recombinant DNA risk assessment tests with dimeric polyoma genomes. Dimeric forms of polyoma DNA, known to be capable of liberating infectious DNA owing to intramolecular recombination, have been completely non-infectious for mice and hamsters when introduced in live *E. coli*. (Martin, Rowe)

Recombinant DNA risk assessment tests with transforming gene of a murine sarcoma virus. Harvey sarcoma virus DNA containing the gene for transformation was completely inactive when introduced into hamsters via live *E. coli*. (Martin, Rowe)

Integration of adeno-associated virus (AAV) DNA. AAV DNA integrates at a few specific sites in host cell genome regardless of virus strain. Some AAV carrier clones carry multiple copies at a single site, arranged in tandem. (Hoggan)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00011-15 LVD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Studies of Small DNA Containing Viruses Belonging to the Family Parvoviridae

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. D. Hoggan	Staff Scientist	LVD	NIAID

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F. Brent Johnson, Dept. of Microbiology, Brigham Young Univ., Provo, Utah.
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LAB/BRANCH
Laboratory of Viral Diseases

SECTION
Viral Oncology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.25	0.25

CHECK APPROPRIATE BOX(ES)
☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Adeno-associated virus (AAV) DNA is integrated at same specific site(s) on host cell genome in different AAV carrier clones regardless of AAV type used to initiate carrier state. It has been found that even though the host integration site(s) are specific, the number of copies of AAV DNA integrated vary from clone to clone and are found integrated in a tandem head to tail fashion. Adeno-associated virus (AAV) structural proteins (VP-1, VP-2, and VP-3) contain extensive areas of sequence homology as analyzed by tryptic and chymotryptic mapping. The new data is consistent with previous experiments suggesting that AAV structural polypeptides either arise from a common large precursor protein or from similar transcripts. Preliminary screening of sera and cerebrospinal fluid (CSF) from schizophrenic patients showed no significant differences in the distribution or titer of antibodies to AAV or other parvoviruses.

Project Description

Objectives: To use molecular biological as well as classic physical-chemical, immunological and epidemiological techniques to elucidate the mechanisms of virus replication, persistence and genetic evolution of viruses belonging to the Family Parvoviridae for better understanding of their natural history and role in disease.

Major Findings

Specific host cell loci serve as AAV integration sites: Additional independent clones of both AAV-1 and AAV-2 carrier cells have been found to give identical AAV integration patterns by using one cut and no cut restriction enzymes to cleave carrier cell DNA and probing Southern blots of agarose analytical gels with probes from full copy AAV DNA as well as specific terminal fragments.

Adeno-associated virus (AAV) structural polypeptide sequence homology. We have previously demonstrated that the structural proteins of AAV possess similar biochemical and immunological characteristics including similar amino acid composition. Using tryptic maps of the various polypeptides we find that almost all of the VP-1 sequences are contained in VP-2 and that the VP-2 sequences are contained in VP-3. Thus AAV are similar to the other parvoviruses in the replication of their constituent polypeptides.

Survey of sera and cerebrospinal fluid (CSF) from schizophrenic patients for the presence of parvovirus antibodies: Thirty eight sera and CSF were tested for the presence of AAV antibodies using complement fixation. No AAV antibodies were found in any of the CSF while about 15% of the sera showed antibody to one or more AAV. All but one serum (97%) were positive for adenovirus group antibody. All sera and CSF were checked for hemagglutination inhibition antibodies against the following hemagglutinating parvoviruses: PVZgd, HS-3, H-1, LuIII, Rat virus (RV) and Minute virus of mice (MVM). All CSF were negative while one serum gave a low titer (1:16) against LuIII. The LuIII was isolated from a latent infection of a human lung cell line but has not been isolated directly from humans or any other animal to date.

Significance to Biomedical Research and the Program of the Institute

The Institute has long supported programs on slow viruses, persistence and their role in disease. Parvoviruses not only provide simple (genomic size less than $2x10^6$ MW) virus models for elucidating basic mechanisms of virus/cell interaction and persistence, they are widespread in nature and may hold the key to our understanding of gene expression. We previously reported that 10% of all human embryonic kidney cultures we have tested contained AAV-2 in an integrated state. What is this genome doing? How is it passed from generation to generation? These and other questions need to be answered if we are to fully understand what role these agents play in disease. For example, it has been shown by at least 2 different groups that cancer patients have significantly lower levels of anti-AAV 2 and 3 antibodies, suggesting these agents provide some sort of protection.

Proposed Course

During the last 16 months our section has devoted over 80% of our time to the study of the molecular biology of retroviruses. The work has been done in collaboration with Drs. W. P. Rowe, J. W. Hartley, and M. A. Martin. This has been done with the view of developing our own expertise in the area of recombinant DNA research so we can more fully exploit the problems of parvovirus research. This activity also provides extra manpower during a time of rapid growth in the retrovirus research area. The Principal Investigator is frequently consulted by others in the parvovirus area and our laboratory serves as a reference laboratory. It is planned to continue our parvovirus collaborative efforts with Berns, Johnson, and Mayor with a small scale in-house effort in this area for about 12 more months before returning to the parvovirus field on a full scale level.

Publications

Lubeck, M.D., Lee, H.M., Hoggan, M.D. and Johnson, F.B.: Adenovirus-associated virus structural protein sequence homology. J. Gen. Virol. 45: 209-216, 1979.

Cheung, A.K.W., Hoggan, M.D., Hausworth, W.W. and Berns, K.I.: Integration of the adeno-associated virus genome into cellular DNA in latently infected human Detroit-6 cells. J. Virol. 33: 739-748, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00012-18 LVD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies of Mouse Leukemia Viruses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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SECTION
Viral Oncology Section

INSTITUTE AND LOCATION
National Institute of Allergy and Infectious Diseases, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
13.6	5.9	7.7

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The natural biology of the complex group of murine C-type RNA viruses is being studied using virological, genetic, and biochemical approaches. Continuing studies of the genetic transmission of both ecotropic and xenotropic virus classes have led to the chromosomal mapping of additional virus-inducing loci. Studies of the recombinant MCF viruses in relation to murine leukemogenesis have delineated certain biological and biochemical correlates of the ability of a given MCF isolate to induce or accelerate leukemia, leading to the recognition of target tissue host range as the major determinant of leukemogenicity. Sensitivity to MCF virus infection has been shown to be under genetic control and the relationship to lymphoid and erythroid neoplasia of MCF virus infection, spread and/or generation is under study. Endogenous virus genomes in various inbred and wild mice are being analyzed using defined biochemical probes derived from molecular cloning of ecotropic, xenotropic and MCF viruses; these include cross-reactive and ecotropic and xenotropic specific viral genome fragments.

PHS-6040
(Rev. 10-76)

This is a long term project of many years duration focusing on the natural biology of C-type viruses of mice. A large portion of the program deals with the genetic transmission of endogenous C-type viral genomes and the generation of novel recombinant viruses that are generally associated with induction of leukemias. The pathogenicity of various isolates of recombinant viruses and non-recombinant (endogenous viruses) is increasingly the subject of investigation. In the past year the use of molecularly cloned fragments of murine leukemia viruses to analyze the structure of endogenous proviral sequences has become a major collaborative program between LVD and the Recombinant DNA Unit in OSD.

Studies relating to genetics of AKR lymphomagenesis. The influence of different mouse strains, serving as partners in matings with AKR, on the incidence and latency of spontaneous lymphoma in F_1 progeny appears to fall into 3 major categories. $Fv\text{-}1^b$ genotype partners, as expected, almost completely suppress spontaneous disease, while mating with most low-virus, low-leukemia $Fv\text{-}1^n$ strains results in a 2-4 month extension of latent period. An exceptional $Fv\text{-}1^n$ strain is DBA/2, whose F_1 progeny with AKR show a markedly reduced lymphoma frequency and much prolonged latency. Basically similar patterns were seen in MCF virus acceleration tests.

In studies carried out by Dr. Cloyd in crosses of AKR (sensitive) and NFS (resistant) mice, sensitivity to MCF virus-accelerated lymphoma was found to be correlated with replication of virus in thymus, which was in turn correlated with presence of endogenous ecotropic MuLV contributed by the Akv-1 and Akv-2 loci from AKR. Evidence was also obtained of an additional independently segregating gene (not H-2 linked) for resistance, which may act by favoring antibody response.

Cells of DBA/1 and DBA/2 mice display a 30-100 fold resistance to MCF virus infection compared to many other inbred mouse strains. This resistance is controlled by a dominant gene, unlinked to Fv-1, and is probably viral envelope determined, since the restriction is not abrogated by addition of helper ecotropic MuLV and pseudotypes of MSV genomes with MCF MuLV are restricted.

Ecotropic MuLV leukemogenesis. Unlike the majority of naturally occurring ecotropic MuLV's, certain isolates from California wild mice, and the so-called laboratory MuLV strains (Moloney, Friend, and Rauscher), can induce leukemias of various forms with latent periods of a few weeks to several months. In mouse strains sensitive to leukemogenesis by these viruses, MCF viruses are regularly detected in preleukemic and leukemic animals. The pathogenicity of these novel MCF isolates, alone and in combination with the ecotropic parent, is under study.

Virology of two unique lines of mice. F/ST is an inbred mouse strain unusual in expressing high titers of both ecotropic and xenotropic MuLV. Studies of genetic transmission of these viruses, in collaboration with Dr. H. Morse, LMI, indicate the presence of several loci for induction of ecotropic MuLV, and a single locus for xenotropic MuLV. In contrast to NZB, the other known high xenotropic virus mouse strain, *in vivo* expression of xenotropic antigen and virus is markedly suppressed in the progeny of matings of F/ST and other inbred strains.

Mus musculus molossinus, the wild mouse of Japan, has also been characterized as a high xenotropic, high ecotropic mouse. Genetic studies have shown this line to be unique in carrying both multiple loci for ecotropic and multiple loci for xenotropic virus induction. Further, preliminary testing of first and second generation backcross mice suggest that the ecotropic virus-inducing loci in this strain fall into both the high and low inducibility categories. Previous studies with high virus inbred mice such as AKR had found only high inducibility loci, while strains with low inducibility locus carried only that one type of locus.

Chromosomal mapping of endogenous MuLV genomes. Further progress has been made on mapping endogenous ecotropic genomes of various inbred mouse strains. Akv-2, the previously unmapped locus of AKR, was found to segregate with chromosome 16 in somatic cell hybrid cell lines. Following on this lead, standard mendelian crosses were carried out using the mahoganoid locus of chromosome 16 as a linkage test, and the Akv-2 locus did show close linkage to mahoganoid. Thus we now know the chromosomal location of all three virus-inducing loci of AKR: the two ecotropic virus loci Akv-1 (chromosome 7) and Akv-2 (chromosome 16), and the xenotropic locus, which is at Bxv-1 on chromosome 1.

The ecotropic virus-inducing locus of SEA/GnJ has been mapped to chromosome 9 by mendelian crosses. This finding brings the number of integration sites for endogenous ecotropic genomes that we have identified in various mouse strains to a total of 8, on 6 different chromosomes.

Use of somatic cell hybrids for studies of C-type viruses. Mouse/hamster interspecific hybrid cell lines have been made from several inbred strains of mice. These lines are being used in several important areas of investigation:

1. Attempts are being made to isolate clonal hybrid cell lines which will support the replication of only one or two of the three major classes of MuLV that can replicate in mouse cells, that is, ecotropic, amphotropic and MCF viruses. This will help clarify whether the various viruses use different cell surface receptors; it may also permit development of specific sensitive cell lines which will support the growth of only one class of virus. Such lines would be of much use for analysis of complex mixed populations of naturally occurring virus.

2. Clones have been isolated that lack the receptor for ecotropic virus but retain an inducible endogenous ecotropic viral genome; these provide for the first time a system for analysis of induction of ecotropic virus in the absence of secondary spread of the infection. By analyzing the time course of virus production following induction of one such cell line we have found that virus production does cut off, as was shown previously for xenotropic virus induction by other laboratories. However, the time required before the cut off occurs is far longer in the case of the ecotropic virus. The mechanism of this important viral shut-off will be investigated.

3. Hybrid lines that retain few mouse chromosomes, in particular chromosomes that carry V-loci or regulatory genes such as Fv-1, are being used for the biochemical studies of the organization of endogenous genomes. These lines offer unparalleled ways of relating specific biologically defined genetic loci to particular nucleic acid sequences and to more accurately determine the association and organization of specific viral sequences in mouse chromosomes.

4. Hybrid cell lines are being used to examine the genetics of XENCSA antigen expression. In this collaborative study with LMI investigators, we have defined 3 levels of XENCSA expression on cell hybrids and have been able to sort chromosomally heterogenous hybrid populations into these 3 classes on the cell sorter. These classes have remained stable for the antigen expression in the subsequent cell passages. Analysis of these three hybrid classes suggest that XENCSA expression is apparently not associated with chromosome 4, in contrast to the inferences from mendelian crosses.

Biochemical analysis of the endogenous murine leukemia virus genomes. A large scale collaborative project has been initiated with Dr. Malcolm Martin of OSD on the use of molecularly cloned probes derived from various murine leukemia viruses to analyse the biochemical nature of endogenous murine leukemia virus genomes. Ecotropic, xenotropic, and MCF viruses cloned in *E. coli* host vector systems have been analyzed for their restriction maps, and a number of subfragments of these genomes have been cloned. These subfragments represent different regions of the viral genome, including portions that are cross-reactive with all members of the group such as the terminal repeat sequences or *gag* gene region, or regions of the *env* gene that are apparently specific for ecotropic and xenotropic viruses. These probes are being used in the Southern blot hybridization system on genomic DNA of various inbred and wild mice following cleavage with various restriction enzymes.

These studies have revealed several important and unanticipated findings. First, they indicate that mice carrying ecotropic genomes have somewhat more proviruses than induction loci. This suggests either that some loci are silent, perhaps defective genomes, or that some of the induction loci may be complexes containing several proviruses in the same region of the chromosome. Second, in the course of analyzing genomic DNA and clones derived from AKR and BALB/c mouse libraries we have observed a striking number of instances where fragments react with both the ecotropic and the xenotropic specific probes. This suggests either that recombinant genomes are common in the mouse DNA or that there are gene regions carrying the DNA of several classes of virus in close association. Third, a number of inbred mouse strains that are negative for infectious ecotropic virus contain an unusual class of DNA sequences that react with the ecotropic specific probe. In some mice these are highly abundant and they have a restriction map quite different from that of the AKR type ecotropic virus.

Risk assessment of recombinant DNA research with *E. coli* K-12. We have continued to collaborate with Dr. Malcolm Martin on the risk assessment experiments involving cloned polyoma virus. A major unanswered question from the previous studies was whether dimeric forms of polyoma DNA, which are known to liberate infectious DNA by intramolecular recombination, might be able to infect animals when introduced in a live *E. coli*. We have tested dimeric clones, supplied by the European polyoma cloning group, in both the mouse and hamster systems. While the hamster tests have not yet been completed, the results in both systems to date have been completely negative. As expected the DNA itself was infectious. A similar hamster test has been done with a Harvey murine sarcoma virus clone in *E. coli K-12*, again with completely negative results.

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RESEARCH HIGHLIGHTS

MICROBIAL SURFACE COMPONENTS: STRUCTURE, FUNCTION, AND CONTROL

Several new projects were initiated to investigate microbial surface components that influence pathogenicity or virulence of various organisms. Studies on gonococcal outer membrane proteins demonstrated "targets" for growth inhibition and killing of these bacteria by proteolytic enzymes (Swanson). Studies on genetic control for expression of a particular outer membrane protein-associated phenotype, i.e., colony opacity, appear consistent with this being a "phase-variation" type mechanism in gonococci (Mayer). Investigation of the contribution of phage infection and phage constituents to the biological/biochemical characteristics of mycoplasma's limiting membrane was undertaken by determining some "biotype" characteristics of mycoplasmas and by demonstrating the "induction" of virulent phages in lysogenic mycoplasmas with uracil, 6-azauracil, or mitomycin (Liss). On the assumption that some virulence-related properties and constituents of *Bordetella pertussis* include outer membrane components, studies were initiated to define and immunochemically or biochemically characterize surface proteins of these human pathogens (Peppler).

PATHOGENICITY AND VIRULENCE-RELATED COMPONENTS OF *BORDETELLA PERTUSSIS*

The virulence of *B. pertussis* is multifactorial and appears to depend on bacterial products or components including "pertussigen" and fimbrial hemagglutinin. Pertussigen was obtained in more highly purified form as crystals (Arai and Munoz). This material induced protection against challenge of mice with *B. pertussis* (Arai and Munoz) and stimulates antigen-specific production of IgE in mice. This "adjuvant" activity of pertussigen can be transferred by inoculation of X-irradiated mice with spleen cells from pertussigen-immunized mice (Sadowski and Munoz). An ELISA was developed for quantifying fimbrial hemagglutinin or anti-fimbrial hemagglutinin antibodies (Arai and Munoz).

STUDIES ON MICROBIAL COMPONENTS THAT INFLUENCE IMMUNOLOGICAL REACTIVITIES

A constituent of mycobacterial cell walls - muramyl dipeptide - has adjuvant actions in diverse model systems. A number of structural analogues of muramyl dipeptide has been synthesized for use in study of structure-function relationships of the adjuvant molecule (Schwartzman). A radiolabeled muramyl dipeptide derivative has been synthesized, but at present the labeled material is not sufficiently stable for utilization in studies on the biological characteristics of this adjuvant molecule (McLaughlin). In combination with muramyl dipeptide (and trehalose dimycolate), endotoxin from Re mutants of *Salmonella* induces regression of transplanted tumors in guinea pigs. This assay has been used to determine the key portions of the endotoxin molecule responsible for such tumor regression (Ribi). Endotoxin was found to stimulate T-lymphocytes in an antigen-specific manner; this reactivity is in addition to a rather non-specific mitogenic effect of endotoxin on B-lymphocytes (Milner and Von Eschen).

A pyridine-soluble component of *Corynebacterium parvum* appears to be active in regressing tumors in mice, and this material appears to lack components implicated in causing splenomegaly and hepatomegaly produced after administration of *C. parvum* whole cells or pyridine-insoluble residue. The pyridine-soluble component of *C. parvum* also caused increased carbon clearance in mice and stimulated proliferative responses in mouse lymphocytes, *in vitro*, as did whole cells. *In vitro* proliferative responses for mouse lymphocytes to T-cell mitogens were depressed by BCG cell wall components. The BCG component also has a negative influence on tumor regression effected by endotoxin + trehalose dimycolate (Cantrell).

BIOCHEMICAL, MORPHOLOGICAL, AND IMMUNOCHEMICAL PROPERTIES OF RICKETTSIAE

Transport and utilization of glutamate or glucose by *Coxiella burnetii* were demonstrated to be optimal at acidic pHs (Hackstadt and Williams). This characteristic may be important for understanding the pathogenicity of this organism which resides and multiplies within phagolysosomes of eukaryotic host cells. *Rickettsia typhi*, which multiplies within host cell cytoplasm, and *Chlamydia psittaci*, whose developmental cycle occurs within phagosomes, both have metabolic pH optima near neutrality. It seems likely that the differing metabolic characteristics of these three intracellular parasites are related to their residence in different compartments of the eukaryotic host cells.

Electron microscopic studies of *C. burnetii* revealed a complex series of morphological changes which seem somewhat analogous to endospore formation (McCaul and Williams). It is suspected that the progeny of this endospore-like formation-sequence are small, compact forms of *C. burnetii* which are highly resistant to destruction by drying, heating, etc.

Also demonstrated for *C. burnetii* were the protective and non-toxic properties of organic solvent extraction residues of whole *C. burnetii* when used as immunogenizing agents in mouse infection models (Cantrell and Williams); this demonstration may have value in development of a more satisfactory vaccine to prevent Q fever in man.

Immunochemical analysis of rickettsiae of the spotted fever group revealed the presence of a common antigen which, when used as a relatively pure immunogen, provoked protection against challenge of mice with *R. rickettsii* (Anacker). This antigen appears to be present on most *R. rickettsii* but is absent from *R. typhi*. These findings have potential relevance to the serologic identification and classification of rickettsiae and to the development of an improved vaccine against Rocky Mountain spotted fever.

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ADMINISTRATIVE REPORT

The Laboratory of Microbial Structure and Function (LMSF) was established during the preceding year and underwent revision in staff during the reporting year. The previously-established three sections (Pertussis, Molecular Biology, and Rickettsial Diseases) now include approximately two-thirds of the scientific staff of LMSF. The remainder have arrived at LMSF during the reporting year and function largely as independent investigators under the general aegis of the Chief. Newly arriving scientists include Dr. Alan Liss, Expert, from the Laboratory of Streptococcal Diseases, NIAID, and Dr. Mark Peppler, Expert, from the Bureau of Biologics, FDA. Joining the LMSF from the University of Utah College of Medicine were Dr. Leonard Mayer, Expert, Dr. Alan Barbour, Senior Staff Fellow, and Dr. John Swanson, Chief. Dr. Ted Hackstadt, Staff Fellow, was added to the Rickettsial Diseases Section. Several members of the technical staff were transferred within LMSF to assist newly-arriving scientists, and Mr. Osmar Barrera transferred to LMSF from the Bureau of Biologics. Extensive renovation of former glassware and media facilities was carried out to house the incoming scientists.

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HONORS AND AWARDS

Journal Editorial Boards:

E. Ribi - Infection and Immunity

J. Swanson - Journal of Bacteriology and Infection and Immunity

Manuscripts from Bacteriological Reviews, Journal of Infectious Diseases, Journal of Immunology, Cancer Immunology and Immunotherapy, European Journal of Cancer, Cancer Research, and Journal of the National Cancer Institute were also reviewed by members of LMSF staff.

Professional Posts:

J. Munoz acted as a trustee of the Stella Duncan Memorial Fund for Allergy Research. He was a Staff Affiliate, University of Montana, Missoula, MT; was elected Chairman of the Immunology Section, 1980-81, ASM; and served as Chairman Elect of the Immunology Section for the current year. He also organized a symposium (Effect of Bacterial Products on Immune Responses) and was co-convener for another session (Cellular Response to Infection) at the annual ASM meeting.

R. Anacker served as co-chairman of the organizing committee for the Conference on Rickettsiae and Rickettsial Diseases to be held at RML, Fall 1980.

J. Cantrell was co-chairman at a session (Tumor Immunology I) of the ASM meeting.

J. Swanson served as a member of the NIH Study Group on Bacterial Virulence and Pathogenicity.

E. Ribi co-chaired a session (Tumor Immunology) at the annual ASM meeting and another session (Biologically Active Fractions) at the 14th Conference on Tuberculosis, U.S. Japan Cooperative Medical Science Program.

A. Liss was appointed Faculty Affiliate, University of Montana, and served as Chairman for the Subcommittee for the Taxonomy of Pseudomonas Bacteriophage, International Committee for the Taxonomy of Viruses.

Honors and Awards (cont'd)

J. Williams served on a Clinical Cancer Program Project Review Committee, NIH, NCI. He co-chaired a session (Intracellular Parasites - Chlamydia and Rickettsiae) at the annual ASM meeting and will chair a session (Biochemistry) in the upcoming RML Conference on Rickettsiae and Rickettsial Diseases.

Invited Lectures:

J. Swanson - University of Rochester Wilson Day Symposium, Rochester, NY.
FEMS Symposium on Microbial Envelopes, Saimaanranta, Finland.
EMBO Workshop on Genetics and Immunobiology of Pathogenic *Neisseria*, Umea, Sweden.
NIH-BoB Symposium on Bacterial Vaccine Development, Bethesda, MD.
Dahlem Konferenzen on Molecular Basis of Microbial Pathogenicity, Berlin, Germany.

E. Ribi - ASM, Miami, Florida.
14th Conference on Tuberculosis, Denver, CO.
International Workshop on Biological Response Modifiers, Bethesda, MD.
1st International Conference on Immunopharmacology, Brighton, England.

A. Liss - 3rd Meeting of the International Organization for Mycoplasmology, Custer, SD.

L. Mayer - EMBO Workshop on Genetics and Immunobiology of Pathogenic *Neisseria*, Umea, Sweden.

J. Munoz - NIH-BoB Symposium on Bacterial Vaccine Development, Bethesda, MD.
Society for the Advance of Chicanos and Native Americans in Science, Albuquerque, NM.

J. Cantrell - International Workshop on Biological Response Modifiers, Bethesda, MD.

Several members of the LMSF staff gave lectures at universities including the University of Montana, Montana State University, Texas A & M University, and the University of California, Berkeley.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00065-07 LMSF-EB

PERIOD COVERED
October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Antigens and Classification of the Rickettsiae

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. L. Anacker	Res. Microbiologist	LMSF NIAID
	R. N. Philip	Medical Director	EB NIAID
OTHER:	E. A. Casper	Nurse Director	EB NIAID

(See same project number in report of Epidemiology Branch)

COOPERATING UNITS (if any) Dr. Karim Hechemy, New York State Dept of Health, Albany, NY
Dr. Catherine Wilfert, Duke Univ Medical Center, Durham, NC

LAB/BRANCH Laboratory of Microbial Structure and Function and Epidemiology Branch, Hamilton, MT 59840

SECTION
Rickettsial Diseases Section and Epidemiology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	1.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☒ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long-range objectives of this project are the development of practicable procedures for classification of spotted fever- and typhus-group rickettsiae and the determination of the nature and biological properties of rickettsial antigens and constituents. Current specific interests are: (1) serotyping rickettsiae by microimmunofluorescence, (2) relationships of rickettsiae by crossed immunoelectrophoresis, and (3) nature and properties of rickettsial antigens determined by other specialized techniques.

Project Description:

A variety of approaches is being utilized to classify rickettsiae of the spotted fever group (SFG) and typhus groups and to isolate and characterize the antigens of these rickettsiae. Included among these procedures are microimmunofluorescence (micro-IF), affinity chromatography, and crossed immunoelectrophoresis (CIE).

Comparisons of SFG rickettsial extracts were made by CIE with intermediate gels containing antisera prepared in rabbits inoculated with formalin-killed, intact sucrose gradient-purified rickettsiae. Two isolates from the United States, Rickettsia rickettsii and R. rhipicephali, one from Africa, R. conorii, and one from Australia, R. australis, appear to share about 3/4 of approximately 15 antigens demonstrated in Triton X-100 extracts of these organisms. All of the rickettsiae possessed the three "major" antigens of R. rickettsii, but each differed with respect to "minor" antigens.

Sera from eight spotted fever patients were tested by CIE for the presence of antibodies to R. rickettsii antigens in a Triton X-100 extract. Several of the sera contained antibody to the same "major" antigen in the extract. Some of the sera reacted with one or more diverse "minor" antigens. These antigens are candidates for further study.

Surface antigens are of primary interest since, presumably, they are the ones which participate in adsorption and penetration of mammalian cells, stimulate the host's immune responses, and react with antibodies in a variety of serological tests. Little is known about the location of the rickettsial antigens thus far demonstrated.

At present, the recognized immunological and biological properties of the SFG rickettsiae can only be attributed to the whole organism rather than to particular surface or subcellular components. We have begun to characterize R. rickettsii antigens purified by a combination of CIE and affinity chromatography to determine the cellular location of antigenic constituents.

In the first phase of the study, an antiserum to a "major" antigen (designated antigen 2) was obtained from rabbits inoculated with immune precipitates prepared by CIE of Triton X-100 extracts of the rickettsiae against rabbit antiserum. This specific serum prevented fever in guinea pigs inoculated intraperitoneally with 10,000 guinea pig ID_{50}, whereas control guinea pigs had fevers ($>40°C$, $\bar{x} = 6.7$ d). Less than 0.001 ml of the same antiserum prevented death in mice inoculated intravenously with 2 LD_{50} of R. rickettsii "toxin." These data appear to show that antibodies to antigen 2 provide significant protection.

Information on the subcellular location of antigen 2 in SFG rickettsiae was obtained with micro-IF studies. Representatives of 9 of the 14 SFG serotypes tested reacted with the antiserum to antigen 2 whereas typhus group rickettsiae did not. It is not clear whether antigen 2 is absent from certain of the serotypes or is "masked" by other surface components in these non-reacting serotypes.

In the second phase of the study, antigen 2 was isolated from an ether extract of purified R. rickettsii by affinity chromatography on a column of Sepharose 4B coupled to the IgG fraction of anti-antigen 2 serum. This preparation of antigen 2, which appeared to be devoid of other rickettsial antigens, protected mice from R. rickettsii "toxin." So far we have been unable to determine the gross chemical composition and protective potency of antigen 2 because the preparation is contaminated with rabbit immunoglobulins eluted from the affinity column with antigen 2 by sodium thiocyanate.

Several lines of evidence suggest that antigen 2 is located on the surface of R. rickettsii. Antibody to antigen 2 "neutralized" viable R. rickettsii injected into guinea pigs and mice, antibody specific for antigen 2 reacted with intact rickettsiae in the micro-IF test, and antibody to antigen 2 agglutinated formalin-killed, intact rickettsiae in the microagglutination test.

Collaborative studies with Dr. Hechemy (development of a latex agglutination test) and with Dr. Wilfert (epidemiological survey of spotted fever in North Carolina) are discussed in Dr. Philip's report.

In the coming year we will concentrate on characterization of SFG rickettsial antigens particularly those which are surface radioiodinated, are stained by immuno-electron microscopy, and react in CIE tests with patients' sera. A different type of affinity column which is not supposed to "leak protein" will soon be tested. The suitability of hybridoma antibodies for affinity chromatographic purification will be determined. Comparisons of recognized species of Rickettsia by CIE will also continue. This study should demonstrate the spectrum of antigens found among the SFG rickettsiae and also suggest which antigens might be used for the preparation of species- and/or type-specific sera.

Publications:

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In press:

Hechemy, K. E., Anacker, R. L., Philip, R. N., Kleeman, K. T., MacCormack, J. N., Sasowski, S. J., and Michaelson, E. E.: Detection of Rocky Mountain spotted fever antibodies by a latex agglutination test. J. Clin. Microbiol.

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PERIOD COVERED

October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Biological Activities of Substances in Bordetella pertussis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. K. Bergman	Scientist Director	LMSF NIAID
OTHER:	J. J. Munoz	Res. Microbiologist	LMSF NIAID
	H. Arai	NIH Visiting Fellow	LMSF NIAID
	J. C. Williams	Scientist	LMSF NIAID

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION

Pertussis Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.2	1.3	0.9

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to study the biological effects of substances from Bordetella pertussis with special emphasis on those substances which induce physiological and immunological responses in experimental animals. Areas of current investigation are: (1) characterization of a highly purified crystalline pertussigen, obtained from B. pertussis, with respect to its ability to induce anaphylaxis, enhance vascular permeability, and produce long lasting histamine hypersensitivity in mice; (2) studies on the ability of pertussigen to enhance the development of acute experimental allergic encephalomyelitis in Lewis rats; and (3) studies on the peptides which occur in the plasma of pertussigen-treated Lewis rats following splanchnic ischemia.

PHS-6040
(Rev. 10-76)

Project Description:

The purpose of this project is to investigate in experimental animals the biological effects of substances from Bordetella pertussis which produce physiological and immunological responses that are related to hypersensitivity and autoimmune phenomena.

Our efforts to test the effects of B. pertussis extracts in vitro on mouse pancreatic β-islet cells were unsuccessful. We could not grow sufficient numbers of β-islet cells in primary tissue culture to conclusively show that B. pertussis extracts induced elevated secretion of insulin.

High performance liquid chromatography (HPLC) was used to determine if the ischemic pancreas of pertussigen-treated rats produces a unique peptide which might be analogous to the myocardial depressant factor (MDF) of Lefer (Fed. Proc. 37: 2734-2740, 1978). Normal and pertussigen-treated rats were sham operated or subjected to splanchnic ischemia for one hour. The peptides in deproteinized plasma samples were measured by HPLC. There were differences in the plasma peptides between control and pertussigen-treated rats; one new peptide was quite prominent in deproteinized plasma from pertussigen-treated rats. It was of low molecular weight and should be isolated and characterized more carefully.

We continued in vivo and in vitro studies on the effects of pertussigen on insulin binding sites of mouse liver cell plasma membranes. Pertussigen-treated mice had more insulin binding sites than control mice. However, when plasma membranes from normal mice were incubated with pertussigen in vitro, there was a decrease in binding sites as compared to untreated membranes. Since pertussigen has such striking effects on insulin secretion, it is important to determine if there are concomitant changes in insulin binding sites.

Drs. Arai and Munoz produced a crystalline pertussigen of very high purity (described in No. Z01 AI 00071-10). We tested the ability of the crystalline pertussigen to produce biological effects in rats and mice. In nanogram quantities, it produced effects similar to those obtained in the past with microgram quantities of crude preparations. For example, it promoted development of active and passive anaphylaxis in mice via its adjuvant and shock enhancing effects and it significantly increased the permeability of the vascular bed in the mouse thigh muscle. The histamine hypersensitivity produced in mice lasted at least 84 days and could be blocked by i.v. treatment with epinephrine. It also enhanced development of acute experimental allergic encephalomyelitis in Lewis rats.

Future work will continue studies on biological effects of crystalline pertussigen and especially on responses which appear to be different from those produced by crude preparations. Crystalline pertussigen is toxic when administered to mice in large doses. The mechanism of death will be studied in these mice. We also want to study the permeability of the vascular bed in pertussigen-treated rats to radiolabeled monocytes.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00071-10 LMSF

PERIOD COVERED
October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Purification and Activities of Pertussigen - a Substance from Bordetella pertussis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. J. Munoz	Res. Microbiologist	LMSF NIAID
OTHER:	P. L. Sadowski	Staff Fellow	LMSF NIAID
	R. K. Bergman	Scientist Director	LMSF NIAID
	H. Arai	NIH Visiting Fellow	LMSF NIAID
	Y. Sone	NIH Visiting Fellow	LMSF NIAID

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION
Pertussis Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.7	3.0	1.7

CHECK APPROPRIATE BOX(ES)
☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER
☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of this project are to fully characterize pertussigen, a substance from Bordetella pertussis, and to study its role in the biological activities of pertussis vaccine. The ultimate goals are to discover the mechanisms of action by which this and other substances from B. pertussis produce various changes in mice and to develop a pertussis vaccine free of undesirable side reactions. The enhanced production of the IgE class of antibodies by pertussigen is of great interest to us. This adjuvant action is important because IgE is responsible for many allergic sensitivities in man and animals. To solve these problems, one of our prime concerns has been to obtain biologically active antigens of B. pertussis in pure form. When this is accomplished, studies on the mode of action of these substances will be greatly facilitated. Another major concern has been to develop assay methods for some of these substances by employing sensitive and reliable methods such as the enzyme-linked immunosorbent assay (ELISA). This test has already been adapted for measuring fimbrial hemagglutinin and its corresponding antibody.

Project Description:

The main objectives of this project are (1) to purify and fully characterize biologically active substances from Bordetella pertussis such as pertussigen and fimbrial hemagglutinin (FHA), and (2) to discover the mechanisms of action of pertussigen. Specifically, we intend to determine how this substance induces protection against B. pertussis infection, how it stimulates production of IgE antibodies, and how it accelerates induction of allergic encephalomyelitis. A practical aim of the project is to develop a safe and non-toxic pertussis vaccine for children.

1. Purification of pertussigen (Munoz, Arai and Sone). Affinity column chromatography was used to purify pertussigen. Culture supernatants containing high histamine sensitizing activity (indicative of presence of pertussigen) were passed through a column of Sepharose 4B conjugated to anti-pertussigen rabbit globulin; pertussigen was retained and could be eluted from the column. Pooled fractions containing pertussigen were concentrated, passed through a Sepharose 6B column to remove large and small molecular weight substances, and pertussigen-containing fractions were pooled and concentrated. From this highly active (1 ng protein per mouse induced histamine hypersensitivity in CFW female mice) solution, crystals of pertussigen were obtained. The crystals were small (about 60 μ long and 3 μ wide for the largest crystals) and probably hexagonal in shape. The crystalline pertussigen has been obtained from two different strains of B. pertussis and by different methods of purification.

Acrylamide electrophoresis (Reisfeld method) of these crystalline materials in solution showed mainly one band compared to many protein bands in the starting material. The yield of pertussigen by this method is low, and this continues to impede our efforts to fully characterize this material chemically. We have found that this material contains all the common amino acids except cystine and methionine. This confirms a previous observation in our laboratory but is at odds with results reported by two other groups who found their preparations to contain cystine and methionine.

2. Activities of crystalline pertussigen (Munoz). Some activities are tabulated in the following table:

Activities of Crystalline Pertussigen in ng per Mouse

Toxicity	(TD_{50})	1000
Histamine sensitizing activity	(SD_{50})	0.4
Mouse protective activity*	(PD_{50})	1700

*Pertussigen had to be detoxified with glutaraldehyde before testing.

Other activities are reported in Project No. 00070-15.

3. Role of pertussigen and FHA on mouse protection (Munoz and Arai). These studies aim to accumulate evidence for or against a role of pertussigen

as the principal mouse protective antigen. Several past observations strongly suggest that pertussigen is an important mouse protective antigen. Recently, Sato et al., Morse et al., and Irons et al. reported that fimbrial hemagglutinin preparations are protective, while pertussigen preparations are toxic. We have confirmed that FHA preparations are protective at about 2-5 µg per mouse, but these preparations are contaminated with pertussigen. We prepared FHA, nearly free of pertussigen, by gel filtration in the presence of Triton X-100. These preparations retained their hemagglutination and immune precipitation activities but failed to sensitize mice to histamine and to protect against infections.

Antisera prepared in rabbits against purified FHA or pertussigen which were known to be specific to the respective substances, a sheep antiserum which contained both anti-FHA and anti-pertussigen, a rabbit anti-pertussigen serum made against a cruder preparation of pertussigen which was free of FHA, and an anti-FHA mouse serum that did not contain anti-pertussigen were tested for their ability to protect mice against i.c. challenge with B. pertussis. Only sera containing anti-pertussigen antibodies protected mice.

4. Enzyme-linked immunosorbent assay (ELISA) for FHA (Arai and Munoz). Estimation of FHA is easily done by the hemagglutinin test, but the measurement of anti-FHA is not. The obvious method of quantitating antiserum by its ability to neutralize the agglutination of erythrocytes by FHA has not worked satisfactorily because of nonspecific inhibitors in normal sera. We have adapted the ELISA for this purpose.

The test for anti-FHA consisted of fixing sheep erythrocytes to the wells of microtiter plates and absorbing FHA onto the erythrocytes. Anti-FHA from a test serum was added at various dilutions and finally antiglobulin serum conjugated to alkaline phosphatase was added. This antiglobulin serum was directed against the species of animal from which the anti-FHA was obtained. We have used mouse, man, and sheep anti-FHA in this test with equally satisfactory results. The enzyme substrate (p-nitrophenol phosphate, disodium) was then added, the plates incubated, the enzyme reaction stopped and the color measured spectrophotometrically at 410 nm. FHA was estimated by fixing sheep erythrocytes to wells and adding various dilutions of FHA, followed by anti-FHA labeled with alkaline-phosphatase, and then substrate. The other steps were done as described above for the anti-FHA test.

Specific anti-FHA can be used as the agent to fix FHA to the wells of microtiter plates.

5. Adjuvant action of pertussigen (Sadowski and Munoz). Pertussigen given to mice as early as 21 days before an antigen was capable of inducing an increased production of IgE antibody (measured by a passive cutaneous anaphylaxis assay) with specificity to the antigen administered at day 0. The optimum interval between administration of pertussigen and antigen is 0-7 days.

SJL mice, which do not respond to antigens with IgE antibodies, become only poor IgE responders following administration of pertussigen. When C57BL/10ScN (responders) and SJL (non-responders) mice are crossed, the offsprings were all responders. Back crosses are now under investigation to see if the response is controlled by a single gene.

To transfer the adjuvant action by cells from pertussigen-treated mice to X-irradiated recipient mice, minute amounts of pertussigen are needed. The pertussigen is tightly bound to the spleen cells, because even ten successive washes of the cells do not remove it. In addition to pertussigen, the lymphocytes must be able to replicate in order to increase the production of IgE.

The important cells involved in the passive transfer of adjuvant activity seem to be the T-lymphocytes, since both B-cells and macrophages can be eliminated from the spleen cell suspensions without decreasing their effectiveness to transfer the adjuvant effect.

Since pertussigen increases insulin production, it has been thought that pertussigen may act through this hormone. However, we have found that pertussigen works well in mice that lack insulin receptors.

6. Miscellaneous experiments

a. Production of crude extracts (Munoz). A great deal of time was spent cultivating cells for the preparation of crude extracts to be used as our starting material for fractionation work. The synthetic medium of Stainer and Scholte was adapted for the production of pertussigen with good results. Pertussigen in culture supernatants was precipitated by $ZnCl_2$, and then extracted by adding Na_2HPO_4 and 50 mM Tris - 1 M NaCl buffer. The titer of these extracts ranged from 1/9000 to as high as 1/35000 as measured by histamine sensitization of mice.

b. Isolation of receptors for pertussigen from erythrocytes (Sone and Munoz). We attempted to solubilize the receptors for pertussigen from human erythrocytes by preparing stromata, then treating them with trypsin, and finally extracting the resultant stromata with 0.5% Triton X-100. The "solubilized receptors" preparation appeared to compete with erythrocytes for pertussigen binding sites. This method may provide partial means of purifying erythrocyte receptors for pertussigen.

Future course. Due to a reduction in the number of staff in the Pertussis Section, our aims for future work will be more limited than in the past. Some aspects of our research will be discontinued. Hopefully, however, we will be able to attract visiting fellows to work on some of the interesting aspects of this project. During the next fiscal year we will concentrate on fully resolving the chemical nature of pertussigen. With the advances made this past year, we should be able to accomplish this goal during the next year. The detoxification of pertussigen will be studied further, and the action of proteolytic enzymes on the activity and structure of this substance will be studied. Much time will be spent producing sufficient quantities of pertussigen for these studies.

Publications:

Arai, H. and Munoz, J. J.: Purification and crystallization of fimbrial hemagglutinin from Bordetella pertussis. Infect. Immun. 25: 460-462, 1979.

Munoz, J. J. and Bergman, R. K.: Biological activities of Bordetella pertussis. In Manclark, C. R. and Hill, J. C. (Eds.): Proceedings of International Symposium on Pertussis. U.S. DHEW, PHS, NIH, FDA, NIAID, CDC, IABS, FIC. DHEW Publ. No. (NIH) 79-1830. Wash., D.C., U.S. Govt. Print. Off., 1979, pp. 143-150.

Arai, H. and Munoz, J. J.: Fimbrial hemagglutinin in stationary and shake-cultures of Bordetella pertussis. Infect. Immun. 25: 764-767, 1979.

Sadowski, P. L., Robbins, K. E., and Munoz, J. J.: Stimulation of IgE by pertussigen. IRCS Med. Sci. 7: 612-1979.

Munoz, J. J., Robbins, K. E., and Cole, R. L.: Reactions in the foot pads of mice induced by pertussigen and endotoxin from Bordetella pertussis. J. Reticuloendothel. Soc. 27: 259-268, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00076-22 LMSF

PERIOD COVERED
October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Mechanisms of Immunopotentiation by Components of Microbes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. A. McLaughlin	Senior Staff Fellow	LMSF NIAID

COOPERATING UNITS (if any) Dr. C. H. Granatek, M.D. Anderson Tumor Institute, Houston, TX; Dr. G. Jones, Syntex Corp., Palo Alto, CA; Dr. L. F. Woodard, Dept Vet Med, U of Idaho, Moscow, ID; Dr. R. K. Sharma, Dept Biochem, U of Tennessee, Memphis, TN.

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Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.3	1.0	1.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to delineate, at the cellular and molecular levels, the mechanism of action of microbial components in immunoregulation. In the past year the direction of the project has changed from elucidation of molecular structural requirements for antitumor activity of synthetic adjuvants to studies of the biochemical mechanisms of action of defined microbial substances and synthetic analogs thereof.

PHS-6040
(Rev. 10-76)

Project Description:

In general, my major objective is elucidating the structural requirements and mechanism of action of immunoregulatory components of microbes. Since considerable progress has been made in the last two years in defining the structural requirements of synthetic adjuvants for immunopotentiating activity, the major thrust of the project is now pertinent to the biochemical mechanism of action of such agents.

Muramyl dipeptide (MDP or adjuvant dipeptide) is a synthetic glycopeptide (N-acetylmuramyl-L-alanyl-D-isoglutamine) which is the minimal structural entity of mycobacterial whole cells responsible for the adjuvant activity of complete Freund's adjuvant. This small molecule and its analogs possess a host of biological activities including adjuvant activity both _in vivo_ (in saline or in oil-in-water emulsions) and _in vitro_. Despite the publication of approximately 50 articles describing biological responses to MDP and certain analogs, only one group has described any studies of the biochemical processes involved in these responses.

A productive and classical means of investigating mechanisms of action of biologically important effector molecules is through the use of radiolabeled ligands. Only with such tools can one make direct measurements of interactions between effector molecules and putative receptors or complexing entities. To elucidate the fundamental processes involved in adjuvant activity, I have undertaken the synthesis of a radiolabeled MDP suitable for ligand-receptor experimentation. The synthesis of a radiolabeled MDP with an anticipated specific activity of approximately 10^3 Curies per millimole is currently in progress. If this proposed radiolabeled molecule is suitable for receptor-ligand studies, considerable interest is anticipated in its use as a molecular probe for the biological phenomenon associated with adjuvant activity.

Because MDP exerts a broad spectrum of biological responses in animals several days following injection of micromolar quantities, I proposed that MDP might act directly on the endocrine system which would in turn produce a cascade effect on the immune system via hormone elaboration. For example, MDP, upon reaching the adrenals, could transiently inhibit production of glucocorticoids. Such a mechanism might explain the immunoregulatory effects of MDP. This hypothesis was tested with the assistance of Dr. R. K. Sharma, University of Tennessee. We measured the capability of MDP and ten analogs to inhibit production of glucocorticoids by ACTH-stimulated rat adrenal cells _in vitro_. No inhibition was demonstrated despite the fact that the rat responds to the adjuvant-stimulatory effects of MDP. Further studies are planned to assure us that inhibition was not observed because of inappropriate experimental design.

Dr. Lynn Woodard, University of Idaho, tested the adjuvant activity of MDP and trehalose dimycolate when combined with whole cells or soluble extracts of avirulent _Brucella abortus_ to protect guinea pigs against challenge with pathogenic B. abortus. We found that both the trehalose dimycolate and MDP were effective adjuvants, and when used in 1% oil-in-water emulsions, they were as effective as complete Freund's adjuvant. Studies are in progress to ascertain the usefulness of such vaccines in preventing abortion in pregnant cattle.

The future course of this project for the ensuing year will be concentrated in elucidation of the biochemical mechanism of action of muramyl dipeptides. The use of a radiolabeled MDP for direct measurement and characterization of putative receptors is one goal. The responses of eukaryotic cells to MDP as measured by modulation of cyclic nucleotide levels, prostaglandin production, protein kinase stimulation, and protease production are currently under investigation or part of proposed studies.

Publications:

McLaughlin, C. A., Schwartzman, S. M., Horner, B. L., Jones, G. H., Moffatt, J. G., Nestor, J. J. Jr., and Tegg, D.: Regression of tumors in guinea pigs after treatment with synthetic muramyl dipeptides and trehalose dimycolate. Science 208: 415-416, 1980.

In press:

Woodard, L. F., Toone, N. M., and McLaughlin, C. A.: Immunogenic properties of soluble antigens or whole cells of Brucella abortus, strain 45/20 associated with mycobacterial adjuvants. I. Soluble antigens. Can. J. Comp. Med.

Woodard, L. F., Toone, N. M., and McLaughlin, C. A.: Immunogenic properties of soluble antigens or whole cells of Brucella abortus, strain 45/20 associated with mycobacterial adjuvants. II. Whole cells. Can. J. Comp. Med.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00077-24 LMSF

PERIOD COVERED

October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Structure and Biological Activity of Endotoxins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. E. Ribi	Res. Chemist (Phys. Chem.)	LMSF NIAID
OTHER:	J. L. Cantrell	Senior Staff Fellow	LMSF NIAID
	S. M. Schwartzman	Senior Staff Fellow	LMSF NIAID
	K. B. Von Eschen	Senior Staff Fellow	LMSF NIAID
	K. Amano	NIH Visiting Fellow	LMSF NIAID

COOPERATING UNITS (if any) Dr. R. A. Parker, Hamilton Biochemical Res. Lab., Hamilton, MT; Dr. K. Takayma, Williams S. Middleton Memorial Veterans Hosp., Madison, WI.

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SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.7	1.9	2.8

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major objective of this project is to correlate chemical composition and structure of endotoxin from polysaccharide deficient Re mutant Salmonella with immunological and non-immunological host reactive properties. The purpose is to fractionate from endotoxic extracts the complex mixture of macromolecular glycolipids, to determine the structure of isolated individual components and to assess the activities of these components. An experimental animal tumor model is used to identify adjuvant active components including those which were chemically modified to minimize toxicity and pyrogenicity. Also, this project continues to perform standard bioassays of endotoxic materials prepared by several members of the LMSF.

Project Description:

This project deals with the structure and function relationship of polysaccharide deficient endotoxic extracts from Re mutant Salmonella. We reported previously that the ability of these extracts to cause regression of transplantable line-10 hepatocarcinoma in syngeneic guinea pigs is synergistically enhanced when inoculated into tumors together with a combination of other "adjuvants" such as mycobacterial cell wall skeleton (CWS), trehalose dimycolate (P3) or synthetic structural analogues of the latter. In last year's progress report, we described that removal of minute quantities of peptidic components, presumably precursor or autolysis products of the peptidoglycan moiety of bacterial cell walls which may have been co-extracted with the endotoxic glycolipid, significantly decreased the ability of endotoxin to bring about tumor regression without affecting endotoxicity. High antitumor activity could be restored to the "peptide-free" partially purified endotoxin (designated B4) by the addition of a nontoxic lipoid side fraction (ACP) recovered during the isolation of endotoxin, which contained a small amount of peptidic substances. TLC of B4 showed it to be a complex mixture containing at least 12 poorly resolved components.

Endotoxin liberated from whole cells by a monophasic solvent consisting of water, phenol, chloroform and petroleum ether according to Galanos et al. has been used by others for most of the more recent structural studies. Based on the work of several investigators, it is postulated that the basic unit is the disaccharide glucosaminyl (6-0-(6')- → glucosamine (glucose amine disaccharide). β-hydroxymyristic acid is linked to the amino group of each sugar unit. At the reducing end, there are three possible components: phosphate, pyrophosphate and 0-phosphoryl ethanolamine. The 0-acyl esters of fatty acids are at the 3 and 4 positions. The 3 position of the sugar at the nonreducing end is occupied by keto-deoxyoctanate (KDO). There can be either a phosphate or a 4-amino-4-deoxy-L-arabinose-1-phosphate at the 4 position. Finally 0-acyl esters of a fatty acid are attached. The fatty acids of the endotoxin are lauric, myristic, polmitic, α-hydroxymyristic and β-hydroxymyristic acids.

Since the structural determinations were done on heterogenous mixtures, the exact structures of any single component of endotoxin are not known. It is clear that by varying the substituent groups at the reducing end, at the 4 position of the sugar, at the nonreducing end, and the 0-acyl fatty acids as well as varying the number of KDO units, one can postulate a large number of possible molecular species. We were not satisfied with the generally accepted view that endotoxic glycolipids are simply "microheterogenic" and that separation of its components differing merely in small structural features is not warranted.

The Galanos-type extracts were neither soluble in water nor in organic solvents. Complete solubilization and disaggregation are required before one is able to effectively fractionate an amphipatic compound like endotoxin. To achieve this, we extracted endotoxin from trypsinized clean cell walls, as determined by electron microscopy, rather than from whole cells. The extract (Fraction I) was treated with 0.5 M sodium acetate (pH 4.5) at 100°C for 30 min, which resulted in removal of at least 96% of the original KDO moiety. The removal of this polar sugar acid and possibly some loss of acid labile phosphate groups, while rendering the reaction product completely soluble in chloroform-methanol (4:1), did not impair the toxicity (lethality for chick embryos, $CELD_{50}$) and pyrogenicity (fever response in rabbits, FI_{40}). This soluble product (Fraction II) was fractionated on a DEAE-cellulose column (acetate form). The column was eluted with a series of solvents in the following order $CHCl_3$ - CH_3OH (4:1), $CHCl_3$ - CH_3OH (7:3), CH_3OH, and finally a linear gradient of 0-0.5 M ammonium acetate in 99% CH_3OH. A sharp peak (medium toxic Fraction III, $CELD_{50}$ 0.46 μg) was observed with the $CHCl_3$ - CH_3OH (4:1) solvent (void volume) and the linear gradient gave a sharp peak (nontoxic Fraction IV, $CELD_{50}$ > 10 μg) followed by a broad peak (toxic Fraction V-VII, $CELD_{50}$ 0.01 - 0.04 μg). The nontoxic Fraction IV also was of low pyrogenicity (FI_{40} = 5 μg) yet, in combination with ACP and P3, afforded regression of and systemic transplantation immunity against the line-10 tumor comparable to that afforded by the original toxic extract (Fraction I). The toxic Fractions V-VII also were powerful pyrogens. The fraction collected in the center portion of the broad peak (Fraction VI) which gave a FI_{40} of 0.001 μg was the most potent pyrogen so far prepared in this and to our knowledge in any other laboratory.

Centrifugal chromatograpy and TLC showed that the nontoxic Fraction IV was devoid of all polar components which were present in chromatograms of the toxic fractions but revealed at least 10 new components in the region of low polarity. The chemical composition of Fraction IV differed from that of Fractions V to VII primarily in that the former contained about 50% less phosphate. In addition, pigmentation indicated that its phosphorylated amino deoxy arabinose may have undergone partial destruction. The removal of the polar phosphate groups would explain the formation of compounds of low polarity. The molar ratios between β-hydroxymyristic acid, normal fatty acids, glucosamine disaccharide and phosphorylethanolamine of Fraction IV were similar to the ratios shown by the toxic Fraction V to VII. β-hydroxymyristic acid invariably is associated with the toxicity of bacterial lipopolysaccharides or glycolipids. Determinations of KDO are commonly used to chemically identify and quantitate endotoxins. Based on chemical analysis, the nontoxic, Fraction IV and the toxic Fractions V to VII were essentially free of KDO. From these results it would appear, superficially at least, that the polar phosphate groups and/or its amino deoxy arabinose derivative need to be linked (as "toxophores") to the acylated glucosamine disaccharide in order to produce a toxic and pyrogenic compound. Difficult fractionation and synthetic work lay ahead to prove whether these assumptions are correct. We note the structural similarity between the mycobacterial adjuvant trehalose dimycolate (P3) in which one molecule of β-hydroxymycolic acid is linked to one hydroxyl group of each monosaccharide (glucose) unit and the components in Fraction IV in which one molecule of β-hydroxymyristic acid is linked to the amino group of each monosaccharide (glucosamine) unit. Based on tumor regression experiments, these two adjuvants acted synergistically as did the non-fractionated original endotoxic extracts in combination with P3. It will be of interest to determine to what extent the nontoxic Fraction IV can replace conventional endotoxin in its immunostimulating activity. For example, is it mitogenic or capable of inducing interferon? Does it remain nontoxic or is its tumor regressive potency abrogated upon presensitization with viable BCG? (See annual report Z01 AI 00087-03, J. Cantrell). Does it act as an adjuvant as determined by a variety of other assays, e.g., stimulate nonspecific resistance to other pathogenic microorganisms or conversely, is it capable of enhancing the susceptibility to infection with non-related pathogens such as _Staphylococcus aureus_?

Publications:

Vosika, G., Schmidtke, J., Goldman, A., Parker, R., Ribi, E., and Gray, G. R.: Phase I-II study of intralesional immunotherapy with oil-attached _Mycobacterium smegmatis_ cell wall skeleton and trehalose dimycolate. _Cancer Immunol. Immunother_. 6: 135-142, 1979.

Vosika, G. J., Schmidtke, J. R., Goldman, A., Ribi, E., Parker, R., and Gray, G. R.: Intralesional immunotherapy of malignant melanoma with _Mycobacterium smegmatis_ cell wall skeleton combined with trehalose dimycolate (P3). _Cancer_ 44: 495-503, 1979.

Ribi, E. E., Cantrell, J. L., Von Eschen, K. B., and Schwartzman, S. M.: Enhancement of endotoxic shock by N-acetylmuramyl-L-alanyl-(L-seryl)-D-isoglutamine (muramyl dipeptide). _Cancer Res_. 39: 4756-4759, 1979.

Ribi, E., Parker, R., Strain, S. M., Mizuno, Y., Nowotny, A., Von Eschen, K. B., Cantrell, J. L., McLaughlin, C. A., Hwang, K. M., and Goren, M. B.: Peptides as requirement for immunotherapy of the guinea-pig line-10 tumor with endotoxins. _Cancer Immunol. Immunother_. 7: 43-58, 1979.

Vosika, G., Schmidtke, J., Goldman, A., Parker, R., Ribi, E., and Gray, G. R.: Phase I study of intradermal immunotherapy with oil-attached _Mycobacterium smegmatis_ cell wall skeleton and trehalose dimycolate. _Cancer Immunol. Immunother_. 7: 221-224, 1980.

In press:

Ribi, E., Cantrell, J. L., Nowotny, A., Parker, R., Schwartzman, S. M., Von Eschen, K. B., Wheat, R. W., and McLaughlin, C. A.: Tumor regression caused by endotoxin combined with trehalose dimycolate. _Sixth International Symposium on Animal, Plant, and Microbial Toxins_, Uppsala, Sweden, August 1979. _Toxicon_.

Ribi, E., Cantrell, J. L., Schwartzman, S., and Parker, R.: BCG cell wall skeleton, P3, MDP and other microbial components--Structure activity studies in animal models. In Hersh, E. and Mastrangelo, M. (Eds.): Augmenting Agents in Cancer Therapy: Current Status and Future Prospects. New York, Raven Press.

Milner, E. C. B., Rudbach, J. A., and Von Eschen, K. B.: Antigen-specific T cell proliferation induced by bacterial lipopolysaccharide. J. Immunol.

Appendix 1. Contract N01 AI 72525. Production of P3 and Isolation, Fractionation, and Purification of Lipopolysaccharides (Endotoxins), Hamilton, Biochemical Research Laboratory, Hamilton, Montana.

Total Man Years: 3 Professional: 0.7 Other: 2.3

Annual Funding: $60,574.00

This project was designed, in part, to study and develop methods for the isolation, fractionation, and purification of bacterial endotoxins. During the reporting year, the contractor has supplied the Laboratory of Microbial Structure and Function with about 500 mg of P3 and about 600 mg of endotoxin which was freed of peptide contaminants (B4) by pressure elution chromatography through columns of microparticulate gel. These refined compounds serve as invaluable baseline materials for numerous tumor immunotherapeutic experiments referred to in four individual annual reports of Laboratory of Microbial Structure and Function.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00078-08 LMSF

PERIOD COVERED
October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Synthesis, Biological Activity, and Immunotherapeutic Activity of Microbial Constituents

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. M. Schwartzman	Senior Staff Fellow	LMSF NIAID
OTHER:	E. E. Ribi	Res. Chemist (Phys. Chem.)	LMSF NIAID
	J. L. Cantrell	Senior Staff Fellow	LMSF NIAID

COOPERATING UNITS (if any) Dr. Werner Brehmer, Robert Koch Institute, Berlin

LAB/BRANCH
Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 2.0	PROFESSIONAL: 0.8	OTHER: 1.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

N-acetylmuramyl-L-alanyl-D-isoglutamine or muramyl dipeptide (MDP) has been shown to be the smallest fragment of the bacterial cell wall capable of acting as an immune adjuvant. The purpose of this project is to chemically synthesize a series of MDP analogs and elucidate their host responsive properties. These compounds will be evaluated in vivo for toxicity, pyrogenicity, tumor regressive properties, as well as protection against airborne infection with influenza virus and virulent tubercle bacilli.

Project Description:

N-acetylmuramyl-L-alanyl-D-isoglutamine or muramyl dipeptide (MDP) was first isolated and synthesized simultaneously by French and Japanese investigators. It was shown to be the smallest fragment of the bacterial cell wall responsible for enhancement of both cellular and humoral immune response.

The main thrust of this project is to chemically synthesize a series of MDP analogs and test their activity in a variety of biological assays. The results of these assays will allow assumptions as to the delineation of fine structure necessary for immunopotentiation by MDP and its synthetic analogs.

As was reported previously (Ribi et al., annual report 1979), our first objective was to determine a shorter synthetic route to MDP. This was achieved in an overall shortening of the synthetic scheme as described in the literature from seven steps to four steps. In all, better than 15 MDP analogs of varying complexity have been synthesized. The number of synthetic reactions necessary to construct their molecular architecture has varied from 4 to 14 separate reactions, each step requiring isolation and purification to homogeneity of the product. Variation of structure centered on modifying amino acid sequence, lipophilicity and the production of monomers suitable for polymerization.

The relative toxicity of these compounds was tested as lethality to chick embryo ($CELD_{50}$) and pyrogenicity for rabbits. All compounds were found to be nonlethal at doses up to 100 μg in chick embryos and of low pyrogenicity as compared to a highly pyrogenic endotoxin (10^5 times less pyrogenic). The desmethyl series appeared to be essentially nonpyrogenic.

Several compounds were found to be effective stimulators of the reticuloendothelial system as measured by carbon clearance assay. In particular, the seryl and α-aminobutyrl analogs were very efficient RES stimulators ($p < 0.001$).

In combination with the microbial adjuvant trehalose dimycolate (TDM), MDP was tested in several assays *in vivo* (line-10 tumor regression, protection assays against influenza virus and tuberculosis). These results could be compared with microbial fractions such as BCG cell walls to determine whether MDP-TDM could substitute for BCG as a model for investigating mechanisms of immunity.

In the line-10 tumor regression system, all MDPs are inactive by themselves, but in combination with TDM, provide an effective modality to regress line-10 tumors providing systemic immunity against tumor rechallenge. The better MDP and TDM combinations (150 μg each) display regression rates of 60-70%, whereas BCG cell walls regress 60% of line-10 tumors treated at comparable doses.

In collaboration with Dr. Werner Brehmer, we are investigating the efficacy of MDP in protecting mice against airborne challenge with influenza virus. Both the alanyl and the desmethyl seryl analogs, in combination with TDM, provided 90% protection. Other analogs were nearly as effective (50-80%). In comparison, BCG cell walls afforded 70% protection.

MDP was also tested in mice against airborne infection with virulent tubercle bacilli (H37RV). Although only the alanyl MDP in combination with TDM has been tested to date, it has shown a greater than 10^3 reduction in viable counts of tubercle bacilli in the lung, approximately the same protection afforded by BCG cell walls. Thus, in these three in vivo assays, MDP analogs + TDM appears to be at least as effective as BCG or nonviable fractions derived from it.

Future studies are projected into improving the efficacy of these compounds. Since MDP is passed from the body extremely rapidly (90% after 30 minutes is excreted intact in the urine), we are now exploring methods of producing MDP analogs that will persist in the body. It is felt that by making MDP more particulate in nature through increasing molecular size, the resulting product will be retained for longer periods in the body and thereby produce a heightened immunoresponse. One of the primary routes being pursued is to synthetically combine the two components MDP and TDM into a single totally synthetic "superadjuvant." Another route which is being investigated is the polymerization of MDP with or without the further attachment of mycolic acid or other TDM substitute to create a totally synthetic peptidoglycan moiety. This would, in effect, be the reverse process of degrading peptidoglycan to form the MDP monomer.

Publications:

In press:

Schwartzman, S. M. and Ribi, E.: A shortened synthesis of adjuvant dipeptide (MDP). Preparative Biochem.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00087-03 LMSF

PERIOD COVERED
October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Immunological Activity of Eukaryotic and Prokaryotic Cellular Components

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. L. Cantrell	Senior Staff Fellow	LMSF NIAID
OTHER:	E. E. Ribi	Res. Chemist (Phys. Chem.)	LMSF NIAID
	S. M. Schwartzman	Staff Fellow	LMSF NIAID

COOPERATING UNITS (if any) Dr. Robert W. Wheat, Duke University Medical Center, Durham, NC; Dr. R. T. Tuttle, Wellcome Research Laboratory, Research Triangle Park, NC; Dr. Jacob Zighelboim, UCLA, School of Medicine, Los Angeles, CA.

LAB/BRANCH
Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 3.0	PROFESSIONAL: 1.0	OTHER: 2.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major objective of this project is to determine the minimal chemical composition and mechanism of action of microbial components responsible for immunopotentiation and immunosuppression. In the past year, attempts were made to identify by fractionation and evaluate by chemical and biological analysis components from Corynebacterium parvum and Mycobacterium bovis (BCG) that are efficacious antitumor agents and/or inhibitors of cell-mediated immune responses. Techniques are being employed to assess the cellular nature of the differentially affected biological responses induced by microbial fractions.

PHS-6040
(Rev. 10-76)

Project Description:

The administration of microorganisms such as Corynebacterium parvum and Mycobacterium bovis (BCG) in animals has been shown to have diverse effects on the immune system. In addition to possessing immunopotentiation properties, these bacteria also have the innate capability of inducing immunosuppression. To date, the specific component(s) of these organisms, which differentially effect the immune response, have not been isolated and purified to homogeneity.

Last year we reported preliminary data indicating that the extraction of whole cells of C. parvum with pyridine, a solvent used to extract glycolipids from bacteria, yielded a pyridine-soluble extract, which when combined with trehalose dimycolate, P3 and synthetic muramyl dipeptide (MDP), induced regression of line-10 tumors in guinea pigs. This year, studies were made to determine the reproducibility of these observations using different lots of the pyridine-soluble extract from two batches of heat-killed C. parvum supplied by Wellcome Research Laboratories. Results of these studies substantiated our preliminary observations in that high cure rates, 74%, were observed in guinea pigs inoculated with emulsions containing a combination of pyridine-soluble extract (300 µg), P3 (50 µg), and MDP (15 µg). Similar cure rates were obtained with each lot of pyridine-soluble extract tested. Although tumor regression was seen in animals treated with MDP (15 µg) + P3 (50 µg) only, the cure rate (30%) was striking less than that observed with the triple combination. The antitumor activity of the pyridine-soluble extract was also evaluated by measuring the extent of tumor growth in BDF_1 mice following a subcutaneous injection of syngeneic L1210 leukemia cells admixed with the extract. Significant antitumor activity, 90 to 95 inhibition of tumor growth, was observed in mice inoculated with 10 to 300 µg of the pyridine-soluble extract. When compared to mice given comparable doses of C. parvum whole cells, significantly more animals were tumor-free following the administration of the extract. Stimulation of the lymphoreticular system was assessed by measuring the degree of splenomegaly and hepatomegaly in mice following an intraperitoneal inoculation of the fractions prepared by pyridine extraction. Marked splenomegaly and hepatomegaly with a concomitant high incidence (40%) of liver necrosis were seen in mice given 100 µg of either whole cells of the extraction residue. In contrast, these pathologic changes were absent in mice inoculated with 600 µg of the pyridine-soluble extract. Collectively, these results clearly show that it is possible to isolate, in soluble form, an antitumor component from C. parvum and suggests that this substance is distinct and separable from those responsible for inducing splenomegaly, hepatomegaly and/or liver necrosis. These findings were reported at the 18th Annual Meeting of the American Association for Cancer Research, May 28-31, in San Diego, CA.

Based on the observation that the soluble extract from C. parvum possessed antitumor activity, current studies, done in collaboration with Dr. R. Wheat, were designed to elucidate at the cellular level the mechanism of this activity. Stimulation of the reticuloendothelial system, as measured by carbon clearance and nonspecific macrophage cytotoxicity, was induced in mice inoculated with either whole cells or the pyridine-soluble extract of C. parvum. A marked increase in the rate of carbon clearance (phagocytic index, $p < 0.001$) was observed in animals treated with the extract. In addition, peritoneal macrophages from mice inoculated with the pyridine-soluble extract were nonspecifically cytotoxic to allogeneic tumor cells in vitro. The kinetics of these parameters paralleled those observed with killed whole cells. These findings demonstrate that both the pyridine-soluble extract and whole organisms induce the same degree of macrophage activation.

A system of in vitro proliferation was used to assess the effects of the pyridine-soluble extract on lymphocyte stimulation. It was found that spleen cells from C57BL/10 mice gave a strong proliferative response upon in vitro stimulation with either whole cells or the pyridine-soluble extract of C. parvum. However, when the response to these agents was compared, distinct differences in the kinetics were noted. In general, the extract was more mitogenic than whole cells requiring a lower dosage and shorter incubation time to induce the maximum response. With the use of athymic, nude mice and enriched populations of T- and B-lymphocytes obtained by nylon wool columns, future studies will be designed to determine whether the extract specifically stimulates T- or B-cells.

Other studies, done in collaboration with Dr. J. Zighelboim, showed that the systemic administration of whole cells or the residue of phenol-water extracted organisms into mice modulated the cell-mediated cytotoxic response to tumor alloantigens. Current studies, using fractions obtained by pyridine extraction, have demonstrated that the inhibition of primary spleen cell cytotoxicity to alloantigens was associated with the extraction residue and not with the pyridine-soluble extract. Attempts to isolate the component in the extraction residue responsible for inhibiting allogeneic CMI are in progress.

Many reports have indicated that the systemic administration of viable BCG in animals induces a state of immunosuppression as measured by enhanced tumor growth and suppressed responses to T-cell mitogens in vitro. However, other reports have shown that the intralesional administration of viable BCG or cell wall components from BCG were efficacious in regressing established line-10 tumors in guinea pigs. Therefore, studies were initiated to determine whether preimmunization with viable BCG or BCG cell wall components would enhance or abrogate the tumor-regressive activity of endotoxin. Strain 2 guinea pigs were given an intravenous injection of viable BCG, BCG cell walls or BCG cell wall skeleton on day zero. Seven days after immunization, the animals were given an intradermal injection of line-10 tumor cells. Six-day-old tumors (10 mm in diameter) were inoculated intralesionally with the combination of endotoxin and P3 associated with oil droplets in saline emulsion. High cure rates (89%) were observed in animals given endotoxin therapy without BCG preimmunization, whereas significantly fewer cures (6%) were seen in guinea pigs presensitized with viable BCG. No abrogation of the antitumor effect of endotoxin was noted in animals presensitized with BCG-cell walls or BCG-cell wall skeleton. Studies were also conducted to assess the effect of systemic administration of viable BCG on the lymphocyte reactivity _in vitro_ to mitogens. Results indicated that spleen cells from guinea pigs treated with viable BCG were refractory (70 to 80% depression of mitogenic activity) to stimulation by T-cell mitogens (Con A or PHA) whereas no decrease in the response to a B-cell mitogen (LPS) was observed. These results suggest an impairment of T-cell functions.

The future course of this project will include (1) attempts to further purify and characterize at the cellular level the immunopotentiating component from _C. parvum_, (2) attempts to isolate and chemically define the component(s) from both _C. parvum_ and BCG responsible for the inhibition of cell-mediated immunity, (3) attempts to characterize, at the cellular level, the mechanism of action of BCG-induced immunosuppression, and (4) attempts to analyze the fractionation products from _C. parvum_ for additional biological activities including the activation of natural killer cells and stimulation of interferon.

Publications:

Cantrell, J. L. and Wheat, R. W.: Antitumor activity and lymphoreticular stimulation properties of fractions isolated from _Corynebacterium_ parvum. _Cancer Res._ 39: 3554-3563, 1979.

Murahata, R. I., Cantrell, J. L., Lichtenstein, A., and Zighelboim, J.: Dissociation of biological activities of _Corynebacterium parvum_ by chemical fractionation. _Int. J. Immunopharmacol._ 2: 47-53, 1980.

In press:

Tuttle, R. L. and Cantrell, J.: _C. parvum_-determinants of biologic activity. In Hersh, E. and Mastrangelo, M. (Eds.): _Augmenting Agents in Cancer Therapy: Current Status and Future Prospects_. New York, Raven Press.

Von Eschen, K. B., Cantrell, J. L., Shaw, M., Stover, P. J., and Ribi, E.: Endotoxin induced suppression of tumor growth in endotoxin responder and nonresponder strains of mice. _Cancer Immunol. Immunother._

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) -	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00182-02 LMSF

PERIOD COVERED
October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Biochemical and Genetical Mechanisms of Obligate Intracellular Parasitism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. C. Williams	Scientist	LMSF NIAID
OTHER:	T. F. McCaul	Expert (Electron Microscopy)	LMSF NIAID
	T. Hackstadt	Staff Fellow	LMSF NIAID
	M. Peacock	Microbiologist	EB NIAID

COOPERATING UNITS (if any) Drs. E. Weiss and G. Dasch, Naval Medical Research Institute, Bethesda, MD; and Dr. J. Wild, Dept. of Plant Sciences, Genetics Section, Texas A & M University, College Station, TX.

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Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION
Rickettsial Diseases Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.8	1.5	1.3

CHECK APPROPRIATE BOX(ES)
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objective is to characterize the biochemical and genetical mechanisms of obligate intracellular parasitism between the eukaryotic host and prokaryotic parasite. Molecular interactions at the cell surface and with soluble components are being investigated as primary determinants of metabolic cooperation between host and parasite. Unusual membrane functions have been discovered which contribute to the success of Rickettsia typhi as a scavenger of critical host components. We have discovered a major determinant of the Q fever agent, Coxiella burnetii, intravacuolar parasitism. Uptake of nutrients and metabolism by C. burnetii is highly dependent upon the hydrogen ion concentration of the phagolysosome which maintains a pH between 4.5 to 4.8. The acidophilic nature of C. burnetii may aid in the development of an axenic medium for the growth of this microorganism. This discovery will have a positive impact on future studies designed to delineate the mechanisms of pathogenicity of this medically important intracellular parasite as well as define an apparently unique response of an obligate intracellular parasite to host cellular defense mechanisms.

Project Description:

The molecular basis of obligate intracellular parasitism is being investigated in a model system employing bacterial species coinjoined to form a model based on compartmentalized growth. These studies encompass those organisms which grow (1) autonomously, (2) extracellularly but in association with eukaryotic cells and (3) intracellularly but within different compartments of the host cell. We are interested in a variety of parasitic mechanisms which may be protective or aggressive responses to host defense mechanisms. The model system employed in our studies exploit the very nature of intracellular parasitism since R. typhi replicate intracytoplasmically and C. burnetii carry out their development in the phagolysosome. Since the highly compartmentalized eukaryotic cell provides a haven for the rickettsiae, the molecular basis of cellular interactions between host and parasite is a fundamental question. Investigations at the biochemical and genetical level should provide information about metabolic cooperation between host and parasite. The functional significance of our metabolism studies may aid the development of an axenic medium for the growth of rickettsiae.

Biochemical studies on rickettsiae require that the bacteria be separated from host components. Previously described purification procedures were modified so that large amounts (50 to 500 mg) of viable C. burnetii could be obtained from infected yolk sacs of embryonated hens' eggs. The procedure involves several differential centrifugation steps and finally banding the bacteria in linear Renografin gradients. The purified organisms were determined to be free of host components by electron microscopy, serologically and biochemically. The procedure also appears to be adequate for R. typhi, Chlamydia psittaci (strain 6BC) and the spotted fever rickettsiae. Organisms purified by our procedures have yielded information not previously possible about metabolism, ultrastructure and surface components of C. burnetii.

Last year we reported on some obligatory features of bacterial functions that had been discovered in purine nucleotide metabolism of R. typhi. This organism carries out its metabolic functions at physiological pH (i.e., pH 7.0) in an environment rich in common substrates such as glutamate and nucleotides. Our published observations suggest that for R. typhi the in vivo activity of adenine interconversion was limited to the nucleotides, with adenosine monophosphate being the end product of adenosine triphosphate catabolism, and that the salvage of purine bases and nucleosides was not an essential feature of purine metabolism. These results elucidate the findings of a previous study which showed that in the absence of glutamate as a source of energy, the adenylate energy charge of resting cells of R. typhi is drastically lowered by the high proportion of AMP.

We have tested the potencies of several antibiotics on the inhibition of C. burnetii, R. typhi and R. rickettsii multiplication in ovo and chicken embryo cell monolayers. The relative effectiveness of the rickettsial inhibitors were evaluated individually and in combination in relation to the selectivity of inhibition. When the antibiotics were administered individually in ovo, the degree of effectiveness for *C. burnetii* was: rifampin>doxycycline> oxytetracycline>trimethoprim; for *R. typhi* and *R. rickettsii*: doxycycline> erythromycin>rifampin>oxytetracycline. Combinations of the effective antibiotics were additive for all the rickettsiae tested. The effective combinations for *C. burnetii* were rifampin plus doxycycline or trimethoprim; whereas for *R. typhi* and *R. rickettsii* they were rifampin plus erythromycin or doxycycline. Surprisingly, erythromycin was not effective against *C. burnetii* in ovo, but it was efficacious in the plaque reduction assay. Some of the antibiotics which were not effective in ovo were shown to inhibit plaque formation at concentrations > 10 μg per ml of medium. Although the antibiotics were effective in increasing the mean survival time and percentage survival of embryos, none of the antibiotics were bactericidal for *C. burnetii*, but they were bactericidal for *R. typhi* and *R. rickettsii*.

More recently we have discovered that acidic conditions activate *C. burnetii* metabolic functions. Intracellular growth of *C. burnetii* and members of the genus *Rickettsia* is compartmentalized into either intravacuolar of cytoplasmic space and thus forms the basis for the separation for the genera. These compartments constitute radically different intracellular microenvironments. The intravacuolar niche occupied by *C. burnetii* is considered a hostile environment to most bacteria due to the number of hydrolytic enzymes, low pH (4.7 - 4.8) and other microbicidal mechanisms. The extracellular metabolic capabilities of *C. burnetii* have been considered minimal when compared to *R. typhi* and *R. prowazeki*. Because of the extreme environment inhabited by *C. burnetii*, we felt compelled to examine the metabolic capabilities of this organism under conditions which more closely reflect that of the intracellular environment. The transport and metabolism of both glucose and glutamate were found to be strictly dependent upon the hydrogen ion environment of the intravacuolar space. Metabolism of both substrates under acidic conditions was augmented up to 1000-fold over rates determined at or about pH 7.0.

Conceptually, the activation of intracellular parasites by the pH of their microenvironment may be a general phenomenon which would regulate energy transformation by vectorial metabolism. To test this hypothesis, we examined the metabolic capabilities of *R. typhi* and *C. psittaci* (strain 6BC). Both of these organisms are representative members of the two orders of *Rickettsias* which have previously been shown to metabolize glutamate. However, *C. psittaci* progresses through its developmental cycle in the phagosome where it prevents phagolysosome formation by localized events. In contrast to *C. burnetii* but in accord with compartmentalized replication, both *R. typhi* and *C. psittaci* metabolized glutamate optimally at about pH 7.0 to 7.4 and neither metabolized glucose to a detectable level at any pH examined. The pH activation of transport and metabolism of an intravacuolar parasite such as *C. burnetii* is logical when viewed in terms of the microenvironment in which the organism replicates. Since lysosomes are known to contain sugars, amino acids and dipeptides, the fusion of phagosomes with lysosomes would provide both nutrients and the proper hydrogen ion concentration for the stimulation of *C. burnetii* metabolism. Recognition of the acidophilic nature of *C. burnetii* may aid in the development of an axenic medium for the growth of this microorganism. The stimulation of metabolism under conditions of intravacuolar pH may explain, in part, the extreme resistance of C. burnetii at neutral pH in extracellular environments. C. burnetii has undoubtedly evolved a number of protective or aggressive responses to the most hostile microenvironment of host defense mechanisms.

In collaboration with Dr. E. Weiss, we carried out studies on metabolic capabilities of Legionella pneumophila. This organism has been grown in axenic media, but it also replicates intracellularly in the vacuole and associated with eukaryotic cell surfaces. Interest in the ability of L. pneumophila to metabolize glucose and glutamate was generated from reports that glucose was not utilized and that serine was the primary energy source. The results of our study suggested that glutamate is utilized primarily as an energy source while glucose may serve as an important metabolite for the nutrition of L. pneumophila.

Studies on the genetic and biochemical characterization of distinct transport systems for uracil, uridine and cytidine in Salmonella typhimurium were published during the past year. A nucleoside-specific system was described which requires a functional uridine kinase as well as a periplasmic binding protein. The transport system is specific for uridine and cytidine and does not appear to contribute significantly to the uptake of deoxythymidine on the purine nucleosides. The transport of uridine is characterized by hyperbolic kinetics with an apparent Km for uridine of 4.3 ± 0.4 μM. Uracil is transported by a completely different system which requires a functional uracil phosphoribosyl transferase and possesses a periplasmic binding protein. Thus, transport of uridine and uracil requires the participation of a periplasmic binding protein and the appropriate enzyme.

The future course of this project will center around four areas as specified below: (1) Since we have discovered the acidophilic nature of C. burnetii, a major portion of our time will be spent on describing the metabolic basis of energy transformation. (2) A thorough search will be made for the proper conditions to grow C. burnetii in an axenic medium. (3) The genetic basis of the phase transition of C. burnetii will be studied with the highly purified microorganisms by attempting to transfer genetic material from phase I cells to phase II cells. (4) Utilization of host nucleotide pools by R. typhi in selected tissue culture strains will be examined. An attempt will be made to correlate rickettsial growth with nucleotide availability.

Publications:

Williams, J. C.: Adenine nucleotide degradation by the obligate intracellular bacterium Rickettsia typhi. Infect. Immun. 28: 74-81, 1980.

Williams, J. C., Lee, C. E., and Wild, J. R.: Genetic and biochemical characterization of distinct transport systems for uracil, uridine and cytidine in Salmonella typhimurium. Mol. Gen. Genet. 178: 121-130, 1980.

In press:

Weiss, E., Peacock, M. G., and Williams, J. C.: Glucose and glutamate metabolism of Legionella pneumophila. Curr. Microbiol.

Hackstadt, T. and Williams, J. C.: Glucose and glutamate metabolism by Coxiella burnetii is activated by intravacuolar pH environment.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00183-02 LMSF

PERIOD COVERED

October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Structural and Functional Relationships of Bacterial Antigens in the Immune Response

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. C. Williams	Scientist	LMSF NIAID
OTHER:	E. E. Ribi	Res. Chemist (Phys. Chem.)	LMSF NIAID
	J. L. Cantrell	Senior Staff Fellow	LMSF NIAID
	T. F. McCaul	Expert (Electron Microscopy)	LMSF NIAID
	T. Hackstadt	Staff Fellow	LMSF NIAID
	M. Peacock	Microbiologist	EB NIAID
	T. A. Damrow	Graduate Student	LMSF NIAID

COOPERATING UNITS (if any) Drs. E. Weiss and G. Dasch, Naval Medical Research Institute, Bethesda, MD, and Dr. C. O. Kindmark, Asst. Head of the Dept. of Infectious Diseases, University Hospital, S-75014 Uppsala, Sweden.

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SECTION

Rickettsial Diseases Section

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 4.5	PROFESSIONAL: 1.7	OTHER: 2.8

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objective is to characterize the antigens of the rickettsiae and other bacteria which are pathogenic for humans. Structure and functional aspects of the immunomodulation by the cell wall and soluble components are currently being analyzed as potential candidates for subunit vaccines. Studies employing chemical extracts of Coxiella burnetii have revealed a particulate component which is nontoxic and provides protection against a lethal challenge in mice and guinea pigs. Soluble antigens have been identified as circulating immune complexes during Q fever infections. Ultrastructural analysis of C. burnetii has revealed endospore formation during the developmental cycle of this highly pathogenic bacterium. This discovery will facilitate future studies since we now have some understanding of the historical relationship between C. burnetii resistance to extracellular environments and its highly infectious capability.

Project Description:

Modulation of the immune response during bacterial infection can produce activation or suppression of a variety of humoral or cellular factors. Components on bacterial surfaces and those secreted during infection have been shown to be responsible for immunomodulatory activities. The objective of this project is the elucidation of the chemical structures required for positive or negative immunomodulatory activities of microorganisms pathogenic for humans. Our goal is the establishment of experimental procedures for analyzing components of bacteria in animal and *in vitro* models. Fractionation procedures involve direct whole cell extractions with organic solvents, separation of cell wall components and analysis of aqueous-soluble fractions. The components of each fraction will be evaluated for immunological efficacy in the mouse, guinea pig and rabbit before subjecting them to clinical evaluation. The ultimate aim is the elucidation of structural and functional relationships between biological response modifiers.

In last year's annual report, we had initiated studies on *Coxiella burnetii*, the agent of Q fever. The illness in humans is clinically characterized by interstitial pneumonia. In addition, endocarditis, pericarditis, and hepatitis have been reported in cases of chronic infections with *C. burnetii*. Although fatalities are rare, histologic analysis of fatal cases has revealed pathologic lesions involving the heart, liver and spleen. Experimentally, laboratory animals such as mice and guinea pigs are susceptible to *C. burnetii* infection. In these animal models, determination of disease is assayed based on serological conversion, febrile response, and/or isolation of the organism from tissues. In humans the appearance of phase I antibodies is diagnostic of chronic Q fever. More importantly, the diagnosis of chronic Q fever is not made until patients present with symptoms of endocarditis.

The purification of *C. burnetii* from host material employing Renografin density gradients was greatly improved during this year. Purified organisms have been shown to be highly infectious and free of host antigens. Organisms purified by the Renografin method have been employed in structural and functional studies.

In collaboration with Dr. Cantrell, studies were designed to evaluate the biological activities and immunoprophylactic capabilities of whole cells (WC) and/or cellular components. Vaccines prepared from formalin-killed whole cells of *C. burnetii* or from chloroform:methanol (C:M) extracts and residues of such cells were examined for their toxicity and immunogenicity in male C57BL/10 mice. Results showed that vaccines containing killed whole cells induced (1) a high incidence of liver necrosis, (2) significant splenomegaly, and (3) significant hepatomegaly in mice. The degree and onset of these pathological changes were directly proportional to the dosage of killed organisms administered. Conversely, no splenomegaly, hepatomegaly, or liver necrosis were observed in mice inoculated with C:M residue or extract. Moreover, killed whole cells were lethal for mice at dosages of 150 to 1200 µg, whereas no deaths were seen in animals given 1200 µg of the C:M residue or the C:M extracts. Moreover, 80 to 90% of mice inoculated with 300 μg of the C:M residue were protected against a lethal challenge of viable rickettsiae, whereas only 50% of the animals given 300 μg of killed whole cells were protected. Treatments with the C:M extract were essentially without value since no antibodies were detectable and low percentage of protection was elicited. Collectively, these results show that, although killed whole cells induced immunity, they were toxic, whereas the C:M residue, which also induced immunity, was essentially nontoxic based on the parameters employed.

Vaccines used in the mouse studies were applied to the guinea pig model. The present Q fever vaccine is a formalin-killed, phase I, whole cell preparation partially purified from the yolk sacs of embryonated hens' eggs. This vaccine causes undesirable side effects described in the mouse model. Vaccine potency of Q fever vaccines has routinely been measured in the guinea pig and expressed as 50% fever suppression dose (FD_{50}) when guinea pigs were challenged with 10^5 guinea pig infectious doses ($GPID_{50}$). The FD_{50} of the whole cell phase I vaccines were in the 0.1 to 0.4 μg range.

In collaboration with Marius Peacock, we have compared and contrasted the whole cell and C:M residue vaccines. Our studies have shown that a better measure of vaccine potency may be the dose of vaccine which will protect guinea pigs against a lethal challenge of _C. burnetii_. Initial experiments have shown the LD_{50} of live purified, Ohio 314 strain of _C. burnetii_, in guinea pigs to be 7.0×10^8 plaque-forming units. The 50% protective dose (PD_{50}) of the C:M residue vaccine against a lethal challenge was found to be 0.71 μg per guinea pig. This PD_{50} dose compares very favorably to the WC vaccine PD_{50} of 0.83 μg. Currently, we are studying the efficacy of Q fever vaccines employing parameters which measure invasiveness into tissues by _C. burnetii_, shedding of the microorganisms and establishment of the chronic Q fever infection.

Ultrastructural analysis of _C. burnetii_ has been carried out in great detail so that we could resolve the apparent discrepancy in the literature about the developmental cycle of Q fever. The combined factors of site of multiplication, resistance to both physical and chemical agents, and transmission by aerosol suggested to us that the developmental cycle of _C. burnetii_ was not at all understood. Electron micrographs of the ultrathin sections of purified rickettsial preparations revealed distinct cell variants with a considerable number of morphological differences. The small cell variant was found to possess a complex internal membrane system, a previously undescribed feature, within the cytoplasm. The membranes were arranged at the periphery of the cytoplasm parallel to the outer membrane. The other large cell variant did not harbor these extensive membranous systems. In contrast to the complex membranous structures, relatively simple intracytoplasmic bodies (80-100 nm) were frequently observed only in the large cell variants by both thin-sectioning and negative staining. The relationship between two distinct morphological variants was investigated further by studying the development of rickettsiae in the chick embryonic yolk sac. The infected yolk sacs were harvested on day 3, 4, 5, 6, 7, and 8 after infection. The microorganisms in 3- to 5-day cultures were prodominantly large cell variants. Small cells did not appear until day 6 after infection. The preparation of day-8 cultures revealed a mixture of degenerated and lysed large cell variants. The small cells also were present, but, in contrast to the large cell variants, no alteration in morphology was detected. Thin sections revealed an electron dense body (100-170 nm in diameter) which was surrounded by a coat of two electron-lucent layers developing within one polar end of the large cell variants. On eventual lysis of the large cell variants, these dense bodies appear to mature to form small cell variants. The relationship between the two distinct morphological cell variants was clarified further by studying the effect of physical conditions on C. burnetii. Suspensions of C. burnetii were subjected to osmotic shock in water followed by sonication, incubation at elevated temperatures and finally centrifugation through a 40 to 70% sucrose density gradient. Examination of the cells by transmission electron microscopy revealed that the final fraction contained rickettsiae of small variant form. The large cell variant was eliminated in the final fraction. The final product was also found to be infectious in chick embryonated eggs. These data suggest that it is the small variant cell of C. burnetii that retains its viability during exposure to extreme environmental conditions.

The appearance of small cell variants during the later stage of infection and the development of daughter cells within the large cell variants suggest that the morphogenesis of C. burnetii is comparable, although not identical, to cellular differentiation of endospore formation in bacteria. The factors involving in the formation and germination of endospores in C. burnetii have not been determined, but previous knowledge of the proliferation and metabolism of C. burnetii provided some insight on the mechanism of spore development. The possession of membranous structures in the cytoplasm and the resistance to physical and chemical agents provide the small cell variants an opportunity both to survive in an extracellular environment during propagation, and to obtain metabolites in an extreme environment of the intracellular vacuoles of host cells. The formation of large cell variants from small cells is considered to result from both the development and changes initiated by enzymatic systems within the intracellular environment of the vacuoles. Induction of endospores may be brought upon by an alteration on the nutritional status of the phagocytic vacuoles. The introduction of the spore formation in C. burnetii developmental life cycle should be advantageous for the microorganisms, and it offers considerable survival value in nature. Q fever is considered an important cause of disease in man, and the knowledge of the biochemical and physiological factors involved in the formation and germination of endospores will lead to further understanding of the host-parasite relationship of C. burnetii.

Immunoelectrophoretic studies carried out on the soluble antigens of C. burnetii have revealed that during infection a circulating immune complex exists which may contribute to many of the clinical manifestations of both the acute and chronic infection. At the present time detection of the immune complex seems to occur by, at least, day 11 postinfection of guinea pigs with 10^5 pfu. Earlier time points postinfection are under study. There are at least five soluble antigens associated with Q fever infection, but we do not know which antigen(s) play a role in the antigen-antibody complex formation.

Surface antigens of C. burnetii are currently being investigated with the aid of ^{125}I-labeling procedures. Preliminary studies have shown that at least six antigens labeled with ^{125}I can be immunoprecipitated with immune sera from guinea pigs and humans. There is, however, a new antigen expressed during chronic Q fever in man. This antigen parallels the appearance of anti-phase I antibody in humans. The usefulness of this antigen in diagnosis of chronic Q fever is under study.

The future course of this project will be continued in three areas as specified below: (1) C. burnetii will be fractionated into cell wall and soluble components. The protective antigens will be localized and separated from toxic factors, and the efficacy of each component will be compared to the whole cell vaccine. (2) Membrane proteins, carbohydrates and other components will be compared in various strains of rickettsiae. Extracellular proteins and glycoproteins will be identified by ^{125}I-labeling patterns and two dimensional chromatography profiles. Carbohydrates will be identified by chemical assay and staining. (3) Early detection of antigens and antibodies will be attempted by employing homogenous antigens and monospecific antisera. Monospecific antisera will be prepared by employing hybridoma techniques. Antibody classes and subclasses induced by various homogenous antigens will be analyzed during immunization as compared to infection carried out in mice and guinea pigs.

Publications:

In press:

Cantrell, J. L. and Williams, J. C.: Toxic and antigenic properties of fractions isolated from Coxiella burnetii. Infect. Immun.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00193-01 LMSF

PERIOD COVERED
October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Gonococcal Surface Components: Structure and Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Swanson	Chief	LMSF NIAID

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 1.3	PROFESSIONAL: 1.0	OTHER: 0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project concerns structure-function relationships of components present on the surfaces of Neisseria gonorrhoeae in an attempt to understand the mediation of biological properties of these organisms related to pathogenesis of gonorrhea. Current work is focused on gonococcal outer membrane proteins as possible sites for action of exogenous proteases. These studies are also directed at understanding the organization of these proteins in the outer membrane of gonococci.

Project Description:

This project aims at defining and characterizing those gonococcal (GC) surface components which are determiners of interactions between these bacteria and host components. Work during the current year has concentrated on outer membrane (OM) proteins in attempts to better understand the properties and organization of these moieties on the GC surface.

Protein I (principal or major OM protein) of the GC OM varies in subunit molecular weight among different strains. Previous studies defined two classes of Protein I from different strains; one class consists of Protein I species with subunit of 34,000 or 33,000 daltons molecular weight and the second class includes 32,000 dalton subunit Protein I species. This classification was based on ^{125}I-peptide mapping techniques. The same method was utilized to investigate another group of OM proteins which are heat modifiable, variably present on gonococci, and recently termed Protein II. Though Protein II species vary in apparent subunit molecular weight in homogenous GC populations and among strains, the ^{125}I-peptide maps of these various Protein II moieties are all quite similar.

The organization of Protein I and Protein II on the organism's surface was probed through use of several proteases. Protein II species are very susceptible to cleavage by trypsin, α-chymotrypsin, or Pronase. Gonococci that bear one or more Protein II constituents are more susceptible to growth inhibition and killing than organisms lacking these OM proteins. The susceptibility of Protein I to proteolysis, in situ, was also studied. Protein I of the 34K-33K class is readily cleaved by α-chymotrypsin or Pronase with 34K species being more susceptible than 33K ones. 32K Protein I moieties are totally resistant to proteolysis by α-chymotrypsin or Pronase.

Gonococci lacking Protein II species vary in their killing and growth inhibition by chymotrypsin depending on the Protein I form borne by the organisms; GC with 34K Protein I are more susceptible than 33K Protein I bearing organisms, and GC with 32K Protein I are resistant to these proteases. The presence of one or more Protein II in the OM confers both enhanced growth inhibition and increased rates of Protein I hydryolsis on intact GC.

Chymotrypsin or Pronase cleavage of Protein I of 34K or 33K molecular weight yields two OM-bound fragments which account for the entire native protein. This suggests that a single cleavage site is exposed on the GC exterior and that the Protein I molecule occurs in the OM as a "hair-pin like" structure.

These studies on GC susceptibility to proteases and the protein targets for protease action on GC yield credence to my previous hypothesis that these enzymes are being potential "selective agents" favoring survival of GC with certain phenotypes in different portions of the female genital tract. Recent studies in another laboratory (Dr. J. F. James, University of California, San Francisco) indicate that susceptibilities of GC to killing by antibody +

complement in normal pooled human sera vary in parallel with their susceptibilities to growth inhibition and killing by exogenous proteases. Stated differently, the results suggest that α-chymotrypsin and antibody + complement recognize similar targets on the GC surface in relation to killing these organisms. Such information may be useful for successful design of a gonococcal vaccine.

Publications:

Swanson, J.: ^{125}I-labeled peptide mapping of some heat-modifiable proteins of the gonococcal outer membrane. Infect. Immun. 28: 54-64, 1980.

Swanson, J.: Adhesion and entry of bacteria into cells: A model for the pathogenesis of gonorrhea. In Smith, H., Skehel, J. J., and Turner, M. J. (Eds.): The Molecular Basis of Microbial Pathogenicity, Dahlem Konferenzen, October 22-26, 1979. Weinheim, Verlag Chemie GmbH, 1980, pp. 17-40.

In press:

Swanson, J.: Gonococcal growth inhibition and hydrolysis of outer membrane proteins by alpha-chymotrypsin. In Danielsson, D. and Normark, S. (Eds.): Genetics and Immunobiology of Pathogenic Neisseria, EMBO Workshop, June 16-19, 1980, Umea, Sweden, University of Umea Press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00194-01 LMSF

PERIOD COVERED
October 21, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Molecular Genetics of *Neisseria gonorrhoeae*

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L. W. Mayer	Expert (Microbiology)	LMSF NIAID

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	1.0	0.4

CHECK APPROPRIATE BOX(ES)
☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
The emphasis of this project is to use molecular genetics, including recombinant DNA techniques, to understand the pathogenesis of gonorrhea. Since the outer surface of the gonococcus has an important role in its ability to cause disease, we are currently studying the genetic control of expression of different molecules in the gonococcal outer membrane. We are attempting to isolate the genes for two major outer membrane proteins, to determine the DNA sequence of these genes and thus predict the primary structures and functions of these proteins. The mechanism of phase transition by which gonococci can alter the composition of their outer membrane is also being studied.

Project Description:

Variations in gonococcal colony morphology related to colony opacity were first described by Swanson and coworkers. The opacity phenotype of isolates from infected persons was a function of the sex of the patient, the anatomical site of isolation, and the time in the menstral cycle of female patients. Sequential isolates from three female patients infected with β-lactamase producing gonococci showed an apparent cyclic change between the opaque and transparent phenotypes. Differences of opaque and transparent phenotypes both in their virulence for chicken embryos and in their attachment to tissue culture cells have also been demonstrated. The evidence cited above suggests a role for the cyclic transition between opaque and transparent phenotypes in the natural history of gonorrhea. This study is being done to further characterize these transitions with the eventual goal of understanding the mechanism of these changes.

To see if any compounds could inhibit the transitions or if any differential susceptibility of the different phenotypes exists, we have screened over 170 compounds and mixtures of compounds. No effect on the rate of transition has been found. We assume that a tenfold change in the rate would be detected by these experiments. Some compounds were more inhibitory for the opaque phenotype, but the differences were small and may not be significant.

The rate of transition was determined for nine strains, and the effect of a few selected alterations in environmental conditions was quantitated. Interestingly, the rate of change was found to be identical in both directions (i.e., opaque to transparent or transparent to opaque). The mean rate of transition ± standard deviation for all determinations was $1.89 \pm 1.08 \times 10^{-3}$/cell/generation. This rate is too high to be mediated by random mutation. The apparent oscilation between two fixed states also suggests that random mutation and reversion is not the mechanism of opacity phenotype transitions.

In addition to identical rates of change for each direction, no differences were observed which correlated with: (a) the state of piliation of the organisms, (b) the number of passages as a particular phenotype, (c) the pH of the growth media from 6.2 to 7.8 or incubation temperature from 30-39°C, (d) the addition of 0.1 - 2 mM $MgCl_2$, 0.1 - 20 mM $CaCl_2$, or mixtures of both, or 0.1 - 20 mM K_2SO_4, or 0.1 mM EDTA, (e) the addition of 25 µg/ml of DNase, or 1-10 µg/ml of DNA isolated from opaque or transparent colony types of gonococci, and (f) there was no association between the loss of the ability to produce pili and the change from one opacity type to another.

The rate of transition and the lack of any effector on the rate suggest that a mechanism of DNA sequence inversion, similar to that which controls Salmonella phase variation, is operating in gonococci. We are presently cloning the gene for the opacity protein and hopefully adjacent control regions. This will not only allow elucidation of the mechanism of transition but will have the bonus of predicting the primary structure of opacity protein from the DNA sequence. E. Gotschlich and coworkers (Rockefeller University) have been unable to sequence this protein because the N-terminus is "blocked."

Due to the cross reactivity of gonococcal membrane proteins, our screening for opacity protein will allow us to detect the cloned gene for protein I or the major outer membrane "porin-like (?)" protein.

Publications:

Tenover, F. C., Mayer, L. W., and Young, F. E.: Physical map of the conjugal plasmid of *Neisseria gonorrhoeae*. *Infect. Immun*. 29: 181-185, 1980.

In press:

Barbour, A. G., Mayer, L. W., and Spratt, B. G.: Mecillinam resistance in *Escherichia coli*: Dissociation of growth inhibition and morphologic change. *J. Infect. Dis*.

Mayer, L. W.: Colony opacity changes in *N. gonorrhoeae*. In Danielsson, D. and Normark, S. (Eds.): *Genetics and Immunobiology of Pathogenic Neisseria*, *European Molecular Biology Organization*, Umea, Sweden, University of Umea Press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) -	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00195-01 LMSF

PERIOD COVERED
October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Molecular Aspects of Mycoplasma-Eucaryotic Interactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Alan Liss	Expert (Molecular Biology and Bacteriology)	LMSF NIAID

COOPERATING UNITS (if any) Univ of Alberta Med Ctr (Canada) (Dr. J. Robertson); Lab of Streptococcal Dis (Dr. R. M. Cole) (Z01 AI 00007-04 LSD); Univ of California, Berkeley Plant Path Dept (Dr. J. Kloepper); Uppsala Univ (Dr. K.-E. Johansson)

LAB/BRANCH
Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 1.0	PROFESSIONAL: 1.0	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Mycoplasma is the common name for a diverse group of cell wall-less procaryotes. Certain mycoplasmas are etiologic agents of plant, animal and human diseases. In humans, Mycoplasma pneumoniae causes primary atypical pneumonia. Some forms of human arthritis and nonspecific urogenital tract infections may also be caused by mycoplasmas. Few details exist defining mycoplasma pathogenicity. This study attempts to characterize mycoplasmas at the molecular level as an essential first step in comprehending host-parasite interactions. Current studies involve characterizing the viruses of mycoplasmas. The carrier state of these viruses and their potential for lysogenic conversion are being investigated. Polyacrylamide gel electrophoresis is being used to compare membrane proteins from infected, uninfected and virus-carrier cells. Interactions between viral, host chromosomal and plasmid DNAs are being characterized by ultracentrifugal and electrophoretic techniques. Tissue culture models will be used to ascertain the ability of infected, uninfected and virus-carrier mycoplasmas to interact with macrophages and other components of the vertebrate immune surveillance system.

Project Description:

Current work involves characterization of the molecular biology and virology of mycoplasma strains.

(1) Nucleic acid metabolism of 17 Acholeplasma strains, 18 Mycoplasma strains, 14 Ureaplasma strains, and 9 Spiroplasma strains was investigated. Preliminary studies tested the growth inhibiting or stimulating properties of 27 nucleotides and their analogues on these strains, either on agar plates or in broth. The Ureaplasma work was done in collaboration with Dr. J. Robertson. All strains were inhibited by 5-fluorouracil (25 μg/ml). In addition, Ureaplasma species were sensitive to 6 of the agents; Mycoplasma species were sensitive to 4 of the agents; Acholeplasma species were sensitive to 7 agents. Further work is in progress.

(2) I am also continuing the characterization of group 1 spiroplasmaviruses which I began under Z01 AI 00007-04 LSD. I have isolated 3 additional group 1 viruses (in addition to the isolate from honey bee BC3, one from honey bee KC3 isolate, one from flower isolate G, and one from corn stunt isolate SERO). These viruses show similar adsorption and thermal inactivation kinetics. They are all resistant to treatment with DNase, RNase, Freon, Triton X-100 and non-idet P40. They are moderately sensitive to ether and very sensitive to chloroform. Spiroplasma hosts selected for resistance to one isolate are subsequently resistant to all of the isolates. Antibodies raised against the SERO isolate also inactivates the other three.

Preliminary DNA-DNA hybridization studies (in collaboration with Dr. L. Mayer, LMSF) show 99% homology between isolates from SERO and KC3. The isolates do show differences in host range patterns and one step growth patterns. All isolates do not kill the cells that they infect.

(3) The carrier relationship of the group 1 mycoplasmaviruses was also studied. In collaboration with Dr. K.-E. Johansson, membrane composition of infected, uninfected and carrier cells was investigated. Uninfected and infected cells appear similar. Cells surviving infection appear to have established a carrier virus state. These cells are now resistant to further infection and have an altered membrane composition. Membrane lipids of the carrier strains showed a large monoglucosyldiglyceride/diglucosyldiglyceride ratio and near absence of diphosphatidylglycerol as compared to sensitive strains.

Viruses of the group 1 type have been induced from 3 A. laidlawii strains. Although not fully defined, it has been possible to induce virus from these cells with uracil, 6-azauracil and mitomycin C. Attempts to induce virus from Spiroplasma, Ureaplasma and Mycoplasma species are planned.

Publications:

In press:

Liss, A. and Cole, R. M.: Spiroplasmaviruses: Group 1 characteristics. Proceedings of the Third Congress of the International Organization for Mycoplasmology.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) -	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00196-01 LMSF

PERIOD COVERED

December 5, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Bordetella pertussis: Outer Membrane Structure and Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. S. Peppler	Expert (Microbiology)	LMSF NIAID

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Microbial Structure and Function, Hamilton, MT 59840

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.8	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Little is known about the surface ultrastructure of Bordetella pertussis. It is the object of this research to define the location, spatial relationships and biochemical composition of the major surface constituents of B. pertussis and their contribution to the disease pertussis. SDS-PAGE of cloned, whole bacterial cells and their cell fractions, coupled with various chemical, immunochemical and enzyme "staining" techniques, as well as ^{125}I-surface labeling, peptide mapping and nearest neighbor (cross-linking) analysis are used to determine the chemical nature, location and relatedness of cell components. Double diffusion and two-dimensional immunoelectrophoresis with hyperimmune and hybridoma antibody are used to follow the qualitative and quantitative production of antigenic constituents in a modifiable defined medium. Purification of the major antigens is sought to chemically define them and to assess their potential in improved vaccines and diagnostic reagents. If possible, the genes coding for important major antigens will be cloned into an appropriate vehicle for enrichment of antigen production.

PHS-6040
(Rev. 10-76)

Project Description:

SDS-PAGE has been used in this project to define the biochemical nature and location of major outer membrane components of various Bordetella pertussis strains. Growth conditions have been shown to greatly affect the banding patterns seen on SDS-PAGE and agar plates made from the defined liquid medium of Stainer and Scholte as modified by Hewlitt et al. have been used to select for naturally-occurring, phenotypically stable "degraded" (non-hemolytic on Bordet-Gengou agar) strains of pertussis. Cloned, isogenic pairs of the hemolytic, virulent wild type and avirulent, non-hemolytic phenotype have been compared on SDS-PAGE and on double diffusion agar plates, to ascertain the growth conditions required for optimal production of virulence-associated components. For example, gyratory-shaken broth cultures favor the growth of the non-hemolytic strains while optimal growth of the hemolytic strain is obtained during growth under static conditions. On SDS-PAGE the non-hemolytic strains are missing at least three peptide bands seen in the hemolytic strains, and aside from losing the ability to produce hemolysis, do not produce agglutinogen 1 or stimulate lymphocytosis in mice. ^{125}I-surface labeling of intact, viable organisms has revealed at least six uniquely "hemolytic" bands and two uniquely "non-hemolytic" bands. The nature of the selection mechanism and the optimal conditions for maintenance of virulence are being determined.

Modification of outer membrane constituents by heat and enzymes have revealed the chemical and organizational relationships of several SDS-PAGE bands. Another unique set of Commassie Brilliant Blue- and Alcian Blue-staining bands show particular sensitivity toward oxidizing agents, apparently due to their association with sugar residues. Their apparent molecular weight increases after exposure to the oxidizing agent.

It is hoped that these studies, expanded to include ^{125}I-labeled peptide mapping and nearest neighbor (cross-linking) analysis will help define the organization, composition and function of the major surface components of pertussis. This information should improve our understanding of the host-B. pertussis relationship, ultimately to improve immunoprophylaxis and diagnosis of this disease.

Publications: None

Laboratory of Persistent Viral Diseases
Rocky Mountain Laboratories
Hamilton, Montana
1980 Annual Report
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Annual Report
Laboratory of Persistent Viral Diseases
Rocky Mountain Laboratories
Hamilton, Montana
National Institute of Allergy and Infectious Diseases
October 1, 1979 to September 30, 1980

RESEARCH HIGHLIGHTS

Anti-leukemia virus hybridoma antibodies produced. Monoclonal antibodies produced by mice recovering from Friend leukemia have allowed precise distinction of Friend-Moloney-Rauscher (FMR) cross-reactive antigens from Friend-Rauscher specific antigens on two virion proteins, gp70 and p15. The biological effects of these antibodies *in vivo* are now being analyzed. Monoclonal antibodies specific for AKR-MCF viruses have also been obtained.

Cell types mediating the Rfv-1 (H-2D) effect on recovery from Friend leukemia have been identified. Nonleukemic cells from spleen, bone marrow or fetal liver of Rfv-$1^{b/b}$ mice mediate elimination of leukemia cells of other Rfv-1 genotypes. Thus, Rfv-1 appears to be an immune response gene controlling kinetics of the leukemia-specific T cell responses.

Leukemia cell variant with abnormal H-2 expression identified. A variant clone of leukemia cells has been found which is not recognized by T lymphocytes with specificity for H-2. H-2 antigens on this clone can be detected with IgG but not IgM antibodies. This clone provides a model for study of the role of expression of H-2 products in H-2-restricted T cell recognition of viral antigens and also should provide information on the influence of the plasma membrane environment on recognition of membrane molecules by T cell receptors and antibodies of various classes.

New type of interferon identified. Mouse peritoneal cells obtained after inoculation of a BCG vaccine were found to produce a unique interferon which protected cells from infection by encephalomyocarditis virus (EMCV). This interferon had some apparent antigenic cross-reactivity with mouse type II interferon, but differed from type II in that it was heat labile and required a longer preincubation time to protect cells from virus infection. This interferon was released by Ia-positive B-lymphocytes present in peritoneal exudates after BCG vaccine inoculation and appeared to be a major *in vivo* protective mechanism against spread of EMCV.

Aleutian disease virus-specific T lymphocyte proliferative response developed in mink. Mink in active stages of Aleutian disease have been found to have a virus-specific T cell blastogenesis response. This response persists until it is suppressed by terminal stages of renal failure, and it appears to be unaffected by the overwhelming virus-specific B lymphocyte response which occurs simultaneously during the disease course. Neither T nor B lymphocyte-mediated immune mechanisms appear to be capable of eliminating the virus infections involving virulent strains. The role of mink genes in influencing the immune responses to different virus strains is now being studied.

DNA and proteins of Aleutian disease virus (ADV) characterized. The ADV genome from a tissue culture strain of ADV has been found to consist of a single noncomplementary strand of DNA of 1.4 X 10^6 dalton size. Single strand nuclease digestion suggested that each molecule contained a stable duplex region capable of self priming.

Sex-limited female protein of hamsters found to be analog of human C-reactive protein (CRP). Hamster serum female protein (FP) was found to be similar to human CRP and amyloid P-component by electron microscopic morphology, N-terminal amino acid sequence, and binding affinity for phosphate esters. The role of FP as an acute phase inflammatory reactant is being studied.

ADMINISTRATIVE REPORT

In the past year Dr. Yoko Arai from the National Institute of Health, Tokyo, Japan has joined our laboratory as a guest worker studying the immune response to rabies virus infection. Dr. James Collins left the laboratory after completion of his third year as a Staff Fellow.

Annual Report
Laboratory of Persistent Viral Diseases
Rocky Mountain Laboratories
Hamilton, Montana
National Institute of Allergy and Infectious Diseases
October 1, 1979 to September 30, 1980

HONORS AND AWARDS

Editorial Boards of Journals:

Dr. B. Chesebro

Associate Editor - *Journal of Immunology*
- *Journal of the National Cancer Institute*
- *Proceedings of the National Academy of Sciences*

Dr. D. L. Lodmell

Associate Editor - *Journal of Immunology*

Professional Posts:

Dr. B. Chesebro - Member, Immunobiology Study Section, Division of Research Grants, NIH; Adjunct Professor - Department of Microbiology, Montana State University, Bozeman.

Dr. M. E. Bloom - Faculty Affiliate, Department of Microbiology, University of Montana, Missoula.

Dr. D. L. Lodmell - Faculty Affiliate, Department of Microbiology, University of Montana, Missoula.

Dr. J. L. Portis - Member, American Association of Immunologists.

Invited Lectures and Participation in Meetings and Symposia:

Dr. B. Chesebro - Invited Participant, Workshop Chairman, 4th International Congress of Immunology, Paris, France.

Dr. J. Coe - Invited Speaker, Symposium - Hamster Immune Responsiveness and Experimental Models of Infectious and Oncologic Diseases, Dallas.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space) -	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00072-09 LPVD

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Host Defense Mechanisms in Viral Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. L. Lodmell	Senior Scientist	LPVD NIAID
OTHER:	Y. T. Arai	Guest Worker	LPVD NIAID

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Persistent Viral Diseases, Hamilton, MT

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major goal of this project is to delineate the mechanisms of host resistance to viral diseases. One model for these studies involves induction of nonspecific resistance of mice to encephalomyocarditis virus by an emulsion of nonviable Mycobacterium tuberculosis, and a determination of the mechanisms for this nonspecific resistance in an in vitro assay system. Additional studies have been initiated to study the pathogenesis and neuroimmunology of rabies virus-infected mice, and to ascertain the mechanism(s) by which mice abort central nervous system infections and recover from disease.

PHS-6040
(Rev. 10-76)

Nonspecific resistance to encephalomyocarditis virus (EMCV) infection. Previous studies have shown that unstimulated peritoneal cells (PC) from mice sensitized with nonviable Mycobacterium tuberculosis inhibited EMCV replication in mouse embryo fibroblast monolayers. PC collected 2 to 6 weeks post-inoculation of mycobacteria were the most effective (>99% inhibition). Inhibition of replication was not detected unless PC were in contact with infected monolayers for a minimum of 8 to 10 hours, and optimal inhibition occurred in cultures infected with a low multiplicity of EMCV. Inhibition of replication was not due to pH changes or depletion of nutrients in cultures, adsorption and/or inactivation of EMCV by macrophages, or killing of monolayers by PC. Inhibition of viral replication was a result of the release of a unique Type II interferon from the mycobacteria-sensitized but unstimulated PC.

As a continuation of this work, we have focused our efforts on characterizing the effector cell from the peritoneal cavity of mycobacteria-sensitized mice that is responsible for inhibiting EMCV replication. It has been determined that the cell adheres to plastic, baby hamster kidney microexudates and nylon wool. The classical mature T lymphocyte is not the effector cell because nylon wool nonadherent cells from euthymic mice are ineffective in inhibiting viral replication, and PC from sensitized athymic nude mice inhibit EMCV replication more effectively than PC from their euthymic littermates. Neutrophils also are unimportant. Furthermore, the effector cell is not a macrophage because nonphagocytic cells which remain after incubation of PC with carbonyl iron or protein-coated silica are as effective in inhibiting viral replication as untreated cells. Additional studies have indicated that incubation of sensitized PC with anti-Ia8 antisera plus C eliminates the cell which inhibits viral replication and produces the unique Type II interferon. Lastly, PC from mycobacteria-sensitized mice have no NK cell activity. The effector cell appears to be an Ia8 + B cell.

Host defenses in rabies virus infection of mice. Projects designed to study the pathogenesis and neuroimmunology of rabies virus-infected mice, and to ascertain mechanisms by which mice abort central nervous system (CNS) infection and recover from paralytic disease have been initiated. We have begun to use the rapid fluorescent-focus-inhibition test (RFFIT) for the titration of neutralizing antibody, and have found the results to correlate quite closely with the in vivo neutralization test in 21-day-old mice. An assay for the quantification of cytolytic antibody has been developed in EL-4 and mouse neuroblastoma cells persistently infected with the ERA strain of rabies virus.

A rabies-specific cell-mediated cytotoxicity (CMC) assay also has been developed. EL-4 cells (H-2^b) infected with ERA virus are more readily lysed by spleen cells from C57BL/10ScN mice (H-2^b) sensitized with ERA virus, than similar mice sensitized with either street, HEP Flury or CVS strains of virus. Maximum CMC occurs 7 to 10 days after either IM or IP sensitization.

Studies with the HEP-Flury strain of rabies virus which is nonlethal for adult mice, but produces death in suckling mice following intracerebral inoculation indicate a marked difference between the two age groups in the occurrence of neutralizing and cytolytic antibodies. Cytolytic antibody appears in the serum of adult mice 6 days prior to that in suckling mice, and optimum serum cytolytic antibody titers of adults exceed that of sucklings by ≥16-fold. Differences in serum neutralizing antibody as assayed by the RFFIT assay were not as marked. Brains from mice of the two age groups contained neutralizing antibody in concentrations similar to that detected in sera, whereas cytolytic antibody never was detected in brains of suckling mice. The importance of the thymus in this model is shown in that the HEP Flury strain of virus killed adult nu/nu mice, but not adult nu/+ or C57BL/10ScN mice.

Preliminary results indicated that with appropriate manipulation, abortive infections occurred in 60% of adult C57BL/10ScN mice inoculated IP with a street virus of bat origin. This unusually high percentage of abortive infections will help to accelerate our understanding of the immune response of these mice and what factor(s) are responsible for their survival in circumstances that usually result in 100% fatality.

The future course of the rabies virus project will focus on understanding the relative roles of humoral and cell-mediated immune responses in recovery from primary rabies virus infections. The incidence of persistent infections in recovered animals and the possibilities of activation to the carrier secretor state will be studied. Emphasis also will be placed on determining if the antigenic differences between various rabies virus strains detected in our CMC assay are also present in the antibody response to these same strains of virus. If so, present vaccines and their effectiveness in certain instances may require reevaluation.

Publications:

Pusateri, A. M., Ewalt, L. C., and Lodmell, D. L.: Nonspecific inhibition of encephalomyocarditis virus replication by a type II interferon released from unstimulated cells of *Mycobacterium tuberculosis*-sensitized mice. *J. Immunol*. 124: 1277-1283, 1980.

In press:

Lodmell, D. L., Cent, Jr., R. T., Pusateri, A. M., and Ewalt, L. C.: Inhibition of encephalomyocarditis virus replication by macrophages from athymic (nude) mice sensitized with nonviable *Mycobacterium tuberculosis*. In Reed, N. D. (Ed.): *Proceedings Third International Workshop on Nude Mice*

Lodmell, D. L. and Portis, J. L.: Immunologic and genetic aspects of Aleutian disease. In Gershwin, M. E. and Merchant, B. (Ed.): *Immunologic Defects in Laboratory Animals*.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00073-15 LPVD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Mechanisms of Immunity and Immunopathology in Virus Related Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. E. Coe	Medical Officer	LPVD NIAID
OTHER:	M. E. Bloom	Medical Officer (Res.)	LPVD NIAID
	R. E. Race	Veterinary Officer	LPVD NIAID
	F. J. Waxman	Senior Staff Fellow	LPVD NIAID

COOPERATING UNITS (if any) Dr. Henry Slayter, Sidney Farber Cancer Institute, Boston, MA; Dr. John Sogn, Laboratory of Immunogenetics, NIAID, Bethesda, MD; Dr. Gregory Printz, Laboratory of Infectious Diseases, NIAID, Bethesda, MD; Dr. S. Suffin, NIAID, Bethesda, MD

LAB/BRANCH
Laboratory of Persistent Viral Diseases, Hamilton, MT

SECTION

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	2.0	2.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Syrian hamster Female Protein (FP) was shown to be a sex limited pentraxin which bound to phosphocholine in Ca^{++} dependent fashion and was structurally homologous to amyloid P component and C-reactive protein. The immunological deficiency of hamster spleen may be explained by a nonspecific suppressor cell which can be separated by differential adherence to nylon wool. The cotton rat (Sigmodon hispidis/NIH) was found to have an inverted IgG$_1$/IgG$_2$ serum concentration ratio, which may explain its unique usefulness in respiratory syncytial virus studies. A mutant strain of Lewis rats was genetically resistant to development of experimental allergic encephalomyelitis although hypersensitivity was present.

This laboratory has previously characterized a fast α globulin detectable by gel diffusion analysis only in adult female hamsters. Substantial amounts (1 to 2 mg/ml) of this sex-limited protein (called Female Protein - FP) are present although its function is unknown. However, it is now apparent that FP is very similar and definitely homologous, with two other proteins, amyloid P component (APC) and C-reactive protein (CRP) previously described in man. Both of these proteins are pentraxins (polygonal appearance by EM) and act as acute phase proteins in man and mouse. APC is also a constituent of amyloid whereas CRP can trigger the complement cascade and may regulate T cells. FP has been shown to be similar to these proteins in that 1) Morphologically, FP appears by EM as a typical pentraxin (collaborative work with Dr. Henry Slayter, Sidney Farber Cancer Institute, Boston, MA. 2) Functionally, FP binds to phosphocholine (PC) in a Ca^{++} dependent fashion (similar to CRP) and can be specifically purified by affinity chromatography utilizing PC-substituted Sepharose and eluting the bound FP with free PC or EDTA. FP will also bind to plain Sepharose in the presence of Ca^{++} (similar to APC) although the yield is less (≃5% of the PC-Sepharose technique). 3) Structurally, the sequence of the 26 amino acids on the amino terminal end (collaborative work with Dr. John Sogn, NIH) was definitely homologous to that of APC in that 19 of 23 identified amino acids (83%) had an identical sequence. APC and CRP have been shown by others to have ≃50% homology. Thus, FP and acute phase proteins of man, rabbit and mouse (CRP, APC) have a common ancestral gene, although FP is sex limited (by testosterone inhibition) and does not appear to act as an acute phase protein at least in female hamsters. The function of FP remains unknown, however, its membership in this interesting family of proteins (pentraxins) points toward potential interactions particularly in the area of amyloid pathology for which Syrian hamster has a noted susceptibility. Preliminary tests of FP effect on hamster T cells have suggested an enhancement of mixed leukocyte response and no apparent effect on mitogen or antigen induced blastogenesis.

A variety of in vitro assays of immunological responsiveness has been developed in the mink and Syrian hamster to answer questions about 1) paucity of suppressor cell activity in ADV susceptible Aleutian genotype mink, and 2) excessive suppressor cell activity in the immunologically incompetent hamster spleen. A direct macrophage migration inhibition assay for production of macrophage migration inhibitory factor has been established and detects hypersensitivity specific for ADV in AD mink or purified protein antigens in immunized mink and hamsters. Suppressor cell activity has not yet been detected with this assay, nor with a primary in vitro antibody synthesis technique. However, a blastogenesis assay has defined a nylon wool nonadherent spleen cell of hamster which was a potent nonspecific suppressor of antigen but not mitogen induced blastogenesis.

In collaboration with Drs. Greg Printz and Steve Suffin (NIAID) we have evaluated the Ig classes of the cotton rat, a uniquely susceptible animal for respiratory syncytial virus (RSV) studies. Differential separation of Ig classes was achieved, and four Ig classes have been defined (IgG_1, IgG_2, IgA and IgM) and specific antisera have been produced. The cotton rat currently in use at NIH (S. hispidis hispidis) has been found to have relatively small amounts of serum IgG_2 (0.2 to 0.6 mg/ml) and approximately 10 times more serum IgG_1 (3.0 to 8.4 mg/ml) in normal and immune serum. This is directly opposite the IgG_1/IgG_2 ratios found in other rodents where serum [IgG_2] predominates (2 to 5X) over [IgG1]. Also, we have evaluated another cotton rat (S. hispidis cienegae), which appears to have a normal serum [IgG2] [IgG_1] ratio. We are currently evaluating various strains of cotton rats to see if the reversed IgG_1/IgG_2 ratio of NIH cotton rat is 1) an isolated genetic event in the closed NIH colony or is a true subspecies mutation, and 2) responsible for the particular susceptibility of this cotton rat for RSV.

A mutant strain of Lewis rats has been shown to have a genetic (dominant, autosomal) resistance to development of experimental allergic encephalomyelitis (EAE) even though hypersensitivity, as assessed by macrophage migration inhibition, was present. Expression of EAE in these animals was possible, however, after passive transfer of lymphocytes from immunized susceptible Lewis rats.

Publications:

Thomas, L. A., Patzer, E. R., Cory, J. C. and Coe, J. E.: Antibody development in garter snakes (Thamnophis spp.) experimentally infected with western equine encephalitis virus. Am. J. Trop. Med. Hyg. 29(1): 112-117, 1980.

In press:

Coe, J. E.: Comparative immunology of old world hamsters - Cricetinae. In Streilein, J. W. (Ed.): The Syrian Hamster: Immune Responsiveness and Experimental Models of Infectious and Oncologic Diseases.

Murphy, F. A., Bell, J. F., Bauer, S. P., Gardner, J. J., Moore, G. J. and Coe, J. E.: Experimental chronic rabies in the cat. Lab. Invest.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00074-08 LPVD

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Host Defense Mechanisms in Chronic Viral and Neoplastic Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. W. Chesebro	Chief	LPVD NIAID
OTHER:	W. J. Britt	Medical Officer (Res.)	LPVD NIAID
	J. K. Collins	Staff Fellow	LPVD NIAID
	M. W. Cloyd	Staff Fellow	LPVD NIAID

COOPERATING UNITS (if any)

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Dr. W. Rowe, Laboratory of Viral Diseases, NIAID, Bethesda, MD
Dr. C. Kozak, Laboratory of Viral Diseases, NIAID, Bethesda, MD

LAB/BRANCH

Laboratory of Persistent Viral Diseases, Hamilton, MT

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 6.5	PROFESSIONAL: 4.0	OTHER: 2.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The main goal of this project is the understanding of host defense mechanisms involved in recovery from virus-induced leukemia in mice. Individual mouse genes have been identified which appear to influence T cell-mediated or humoral virus-specific responses and the interactions of these mechanisms have been studied. Monoclonal antibodies specific for various viral proteins have also been selected and the biological roles of antibodies of different specificities and Ig classes are being studied both *in vivo* and *in vitro*.

PHS-6040
(Rev. 10-76)

Spontaneous recovery from Friend virus-induced erythroleukemia occurs in some mouse strains. This recovery is influenced by several different mechanisms controlled by mouse genes. We have studied the mechanisms and interactions of two genes, Rfv-1 and Rfv-3.

Influence of the Rfv-1 (H-2D) gene. Rfv-1 is located in the D region of the H-2 complex and appears to influence the Friend virus-specific cell-mediated immune response. Our most recent experiments indicate that this gene operates through its expression in nonleukemic cells of the spleen, bone marrow and fetal liver. Thus, the main effects of this gene do not function via expression of H-2D antigens on target leukemia cells, but instead appear to influence kinetics of development of the virus-specific T cell proliferative and cytotoxic responses *in vivo*.

Influence of the Rfv-3 gene. The Rfv-3 gene is a non-H-2 linked gene whose chromosomal location is presently unknown. Recent attempts to establish linkage with several isozyme markers were unsuccessful. The Rfv-$3^{r/s}$ genotype is necessary, but not sufficient, for recovery from leukemia. This gene appears to influence the humoral immune response to FV-induced antigens. In mice which develop this antibody response, but fail to develop concomitant cell-mediated immunity, leukemia persists until death, but virus expression by leukemia cells is drastically reduced. Our recent evidence indicates that both gag (p15) and env (gp70) proteins of the ecotropic F-MuLV helper virus are reduced or eliminated from the leukemia cell surfaces. Some intracellular viral proteins are also reduced. Furthermore, on these cells a new gp70 has been found which appears to be derived from recombinant (MCF) viruses. All these effects are dependent on the presence of mouse antibody to FV and are reversed when cells are transferred to an antibody-free host.

Persistence of virus infection. Mice which recovered from FV leukemia were found to have low levels of persistent virus infection consisting of both helper F-MuLV and defective SFFV. It is currently unknown whether the infected cells are transformed leukemia cells or not. Nor is it clear what host defense mechanisms are preventing proliferation of infected cells.

Production of antiviral hybridomas. In order to study the effects of mouse antibodies in the FV system, we have produced a group of monoclonal antibody secreting hybridomas with specificity for erythroleukemia cells. Most hybridomas are directed at the viral proteins gp70, p15 and p15E; however, some react with nonvirion proteins. Two hybridomas, one anti-gp70 and one anti-p15, have the FMR pattern of reactivity. However, most are specific for Friend or Rauscher viruses only.

Recently hybridomas have also been made against AKR MCF virus. MCF-specific as well as ecotropic-specific clones have been isolated, although the virus-induced proteins recognized by these antibodies have not been identified.

Viruses involved in naturally-occurring leukemia. We have shown that certain subclasses of MCF virus appear to be the oncogenic viruses in high leukemic AKR mice, whereas the other classes of MuLV's are not leukemogenic. In genetic studies with AKR mice, the MCF virus was shown to collaborate with endogenous ecotropic virus in causing leukemia. The ecotropic virus conveyed permissiveness for replication of the MCF virus.

An MCF isolate from an Akv-1 congenic mouse was previously shown to be nonleukemogenic in the AKR acceleration test and have RNA oligonucleotide fingerprints characteristic of nonleukemogenic MCF viruses, but it replicated to a moderate extent in the thymus of inoculated mice. This MCF when phenotypically mixed with ecotropic virus became leukemogenic for AKR mice. Recovery experiments revealed that replication of the MCF virus was enhanced. Thus, leukemogenicity correlated with extent of replication and not with oligonucleotide patterns.

Leukemogenicity by MCF viruses is not only strain specific, but these MCF viruses also demonstrate tissue tropism. The leukemogenic AKR MCF's only replicate in the thymus in vivo. The target cell for replication was found to be a cortisone-sensitive subpopulation of immature Thy 1-positive T lymphocytes. The tumors induced are large-cell lymphoblastic lymphomas expressing Thy-1 and varying amounts of Lyt-1, Lyt-2 and MCF specific antigens, but do not express TdT. In contrast, MCF viruses isolated from NFS mice congenic for a C58 ecotropic virus locus are splenotropic. The target cell appears to be a B cell as judged by lack of Thy-1, nonadherence to plastic, and positive adherence to nylon wool.

Host genes regulating MCF leukemia. Genetic analysis of susceptibility or resistance to MCF MuLV induction of leukemia was studied in crosses between AKR (susceptible to MCF) and resistant strains. In addition to the ecotropic virus loci (Akv-1 and Akv-2) which rendered susceptibility, a gene(s) responsible for a humoral immune response conveyed resistance to leukemia development. This resistance appeared to be overridden by a double gene dose of ecotropic virus.

In crosses with C58/Lw, a gene closely linked to Fv-1 was found to be responsible for resistance to MCF leukemia although the Fv-1 types of the parental mice were thought to be identical. This may indicate that Fv-1 (known to control replication of MuLV's) may be more polymorphic than previously realized. Studies using a number of crosses are in progress.

Publications:

Doig, D. and Chesebro, B.: Anti-Friend virus antibody is associated with recovery from viremia and loss of viral leukemia cell surface antigens in leukemic mice. Identification of Rfv-3 as a gene locus in influencing antibody production. J. Exp. Med. 150: 10-19, 1979

Chesebro, B., Bloom, M., Wehrly, K., and Nishio, J.: Persistence of infectious Friend virus in spleens of mice after spontaneous recovery from virus-induced erythroleukemia. J. Virol. 32: 832-837, 1979.

Chesebro, B., Wehrly, K., Doig, D. and Nishio, J.: Antibody-induced modulation of Friend virus cell surface antigens decreases virus production by persistent erythroleukemia cells: Influence of the Rfv-3 gene. Proc. Natl. Acad. Sci. USA 76: 5784-5788, 1979.

Collins, J. K. and Chesebro, B.: Spontaneous cessation of virus production by a Friend virus leukemia cell line: overgrowth by nonproducer cell clones. J. Natl. Cancer Inst. 64: 1153-1159, 1980.

In press:

Britt, W. J. and Chesebro, B.: Recovery from Friend virus leukemia is determined by the H-2 genotype of nonleukemic cells of the spleen and bone marrow. In Skamene, E. (Ed.): Perspectives in Immunology.

Chesebro, B.: The influence of the major histocompatibility complex (H-2) on oncornavirus-induced neoplasia in mice. In Kaiser, H. E. (Ed.): Comparative Pathology of Abnormal Growth.

Collins, J. K., Britt, W. J. and Chesebro, B.: Cytotoxic T lymphocyte recognition of gp70 on Friend virus-induced erythroleukemia cell clones. J. Immunol.

Collins, J. K. and Chesebro, B.: Synthesis, processing and cell surface expression of Friend and xenotropic murine leukemia virus gp70 antigens on Friend virus-induced erythroleukemia cell clones. J. Immunol.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00085-03 LPVD

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Biology of Aleutian Disease Virus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. E. Bloom	Medical Officer (Res.)	LPVD NIAID
OTHER:	J. E. Coe	Medical Officer	LPVD NIAID
	R. E. Race	Veterinary Officer	LPVD NIAID

COOPERATING UNITS (if any)

Dr. John Gorham, Washington State University, Pullman, WA
Dr. David Porter, UCLA School of Medicine, Los Angeles, CA
Dr. David Ward, Yale University School of Medicine, New Haven, CT

LAB/BRANCH

Laboratory of Persistent Viral Diseases, Hamilton, MT

SECTION

INSTITUTE AND LOCATION

NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 2.0	PROFESSIONAL: 1.0	OTHER: 1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goal of this project is to study Aleutian disease virus (ADV), a parvovirus that causes a persistent viral infection of mink associated with immune complex disease. We have used a cell culture adapted strain of ADV to characterize the structure of the viral genome and coat capsid. This information will be used to determine 1) What viral antigens are involved in the generation of immune complexes. 2) What the level of virus expression is in persistently infected animals.

We have previously shown that an isolate of Aleutian disease virus (ADV), adapted to growth in Crandall feline kidney cells, could be satisfactorily purified to homogeneity and radiolabeled with DNA precursors. In the past year we have defined the viral genome to be that of a parvovirus and have begun characterization of the viral capsid.

The genome of radiolabeled ADV DNA has been studied using sucrose gradients and agarose gel electrophoresis. Our results showed that the ADV genome was a single noncomplementary strand of DNA approximately 1.4 to 1.5 X 10^6 daltons. Experiments with single strand specific nuclease digestion and in vitro DNA synthesis yielded results consistent with a largely single stranded molecule that contained a stable duplex region capable of initiating self-primed DNA synthesis at the 3' end.

Our plans in collaboration with Dr. David Ward are 1) To further delineate the genome fine structure, 2) To identify the replication form of ADV DNA, and 3) Possibly to use recombinant DNA technology to develop reagents suitable for analyzing genome expression in situ.

Initial study of ADV proteins using SDS-polyacrylamide gel electrophoresis of ^{35}S-methionine labeled virus has shown that ADV contains two major structural polypeptides with molecular weights of 89,000 and 77,000 daltons and that infected cells also contain a 72,000 dalton species. We plan to confirm these results and to utilize immunoprecipitation to define 1) What viral proteins host antibodies are directed against, and 2) Whether immunoglobulins of all classes contain antiviral specificity. In addition we plan to radiolabel proteins in short term cultures of infected mink tissue to ascertain what viral proteins are synthesized in vivo.

Publications: None

In press:

Bloom, M. E., Race, R. E. and Wolfinbarger, J. B.: Characterization of Aleutian disease virus as a parvovirus. J. Virol.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 AI 00086-03 LPVD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Mechanisms of Immune Recognition of Viral Antigens in Persistent Viral Diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major goal of this project is the definition of mechanisms of immune recognition of viral antigens on the cell surface of virus-infected cells. We are using cell lines persistently infected with murine leukemia viruses as our primary model system. FY7, a cloned variant of the ($H-2^b$) murine leukemia FBL-3, has been found to express $H-2K^b$ and $H-2D^b$ antigens recognized by antibody but not by T lymphocytes. Biochemical analysis has shown that these H-2 products of FY7 are expressed in an unaltered form. Properties of the cell membrane and perhaps the orientation of these antigens in the membrane of FY7 appear to determine the capacity of these cells to be recognized by T lymphocytes. This lack of CTL recognition can be reversed by passage of FY7 in vivo in the ascites form. This feature of FY7 is mimicked by the interaction of IgM but not IgG anti-H-2 antibody with this cell line. We are currently studying this phenomenon using monoclonal antibodies to cell membrane antigens of FY7 with the intention of quantifying antigenic sites on the cell membrane and the binding constant of the antigen-antibody interaction.

T lymphocyte recognition of cell membrane antigens. FY7, a cloned variant of the $H\text{-}2^b$ murine leukemia, FBL-3, is highly immunogenic when injected s.c. into syngeneic mice. Cytotoxic T lymphocytes (CTL) from these mice lyse FY7 grown in the ascites form (FY7As) but fail to lyse FY7 propagated *in vitro* (FY7Tc). We have found that FY7Tc is also not recognized by anti-H-2 cytotoxic T lymphocytes (CTL) although FY7Tc and EL-4 (both $H\text{-}2^b$ leukemias) express comparable amounts of $H\text{-}2K^b$ and $H\text{-}2D^b$ on their cell membranes. FY7Tc differs from EL-4 in that it fails to competitively inhibit the lysis of $H\text{-}2^b$ targets by anti-$H\text{-}2K^b$ CTL. Biochemical analysis using immunoprecipitation and resolution by both SDS PAGE and two dimensional PAGE have revealed no demonstrable alterations in $H\text{-}2K^b$ product of FY7. Thus, although $H\text{-}2K^b$ is expressed on the membrane of FY7 in an unaltered form, it is not recognized by CTL. There are reports of similar dissociation between the recognition of H-2 by antibody and CTL in a subline of the $H\text{-}2^d$ mastocytoma, P815, and a variant of the $H\text{-}2^d$ myeloma MOPC-315. The combined evidence suggests that properties of the cell membrane may determine the capacity of a cell to be recognized by antigen receptors of T lymphocytes. Recent work from our laboratory indicates that although IgG anti-$H\text{-}2K^b$ and $H\text{-}2D^b$ antibodies bind to the cell membrane of FY7Tc, IgM antibodies of the same specificity fail to bind. There appears, therefore, to be a correlation between the lack of recognition of H-2 antigens on FY7Tc by CTL and IgM antibody. It is conceivable that the binding constant of the T cell receptor is very low (i.e., as is the case for the K_a of IgM). The T cell may manifest its antigen reactivity due to multivalent binding. Qualitative features of the expression of H-2 antigens in the membrane of FY7Tc may not allow sufficient multipoint binding for stable CTL or IgM interaction, thus preventing immune recognition. Since FY7As is recognized by both CTL and IgM antibody, we plan to compare the antigen expression of FY7Tc and As. In collaboration with Dr. Chesebro, we will be using monoclonal antibodies of different classes directed to the same membrane antigen. Preliminary studies, using an ^{125}I-labeled IgG_{2a} anti-Friend gp70 hybridoma, show its specific reactivity with the gp70 of FY7. Using this reagent, we are attempting to measure the number of gp70 molecules on the cell surface of FY7Tc and As and to estimate the K_a of the Ag-Ab interaction. In this way, we will be able to document the relative importance of quantitative versus qualitative differences in antigen display which might account for the lack of recognition of FY7Tc by T thymphocytes.

We have evidence which suggests an alteration in some properties of the cell membrane of FY7Tc. Electron microscopic studies indicate that FY7As has C-type virus budding extensively over most of the cell surface, whereas FY7Tc reveals no virus budding from the surface. Instead virus appears to be budding into intracytoplasmic vacuoles of FY7Tc, and this virus is released into the extracellular compartment as large clumps of virions by a process akin to exocytosis. The nature of this apparent cell membrane abnormality is the subject of continuing study with particular emphasis on its role in antigen display at the cell surface.

In vivo studies. FBl-3 is generally considered a highly immunogenic neoplasm which when injected subcutaneously into syngeneic recipients produces transient palpable tumors. We observed, however, that FBL-3 manifests a malignant course if the cells prior to s.c. inoculation were grown for an extended period (14 days) in the peritoneal cavity. With the aid of a summer student, this change in in vivo behavior of FBL-3 has been further analyzed. We found that out of 11 clones of the original parent line one clone expressed a malignant phenotype. Thus, the change in behavior of FBL-3 appears to be due to selection of preexisting malignant variants. Of interest, however, was that this malignant clone had an unusually extended latent period. Palpable tumors were not detected until 2 to 3 weeks after s.c. inoculation, whereas with those clones having a benign course, tumors appeared within 5 to 10 days. These findings suggest that the in vivo behavior of the uncloned tumor cell population may be determined by the relative tempo of expansion of benign and malignant subpopulations. Effective immunization may occur only if early expansion of benign subpopulations occurs prior to the appearance of those cells having the malignant phenotype.

Publications:

Portis, J. L. and Coe, J. E.: Deposition of IgA in renal glomeruli of mink affected with Aleutian disease. Am. J. Pathol. 96: 227-234, 1979.

Portis, J. L. and McAtee, F. J.: Absence of recognition of tumor associated antigens on a cloned murine leukemia cell line is associated with lack of H-2D^b expression. In Yohn, D. S., Lapin, B. A. and Blakeslee, J. R. (Eds.): Advances in Comparative Leukemia Research 1979. Elsevier/North Holland, 1979, pp. 155-156.

In press:

Portis, J. L. and McAtee, F. J.: Dissociation of H-2 recognition by antibody and cytotoxic T lymphocytes on a cloned murine leukemia cell line. Immunogenetics.

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Immunobiology of Aleutian Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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SUMMARY OF WORK (200 words or less - underline keywords)

Aleutian disease is an immune complex mediated, parvovirus caused infection of mink. Preclinical incubation periods measured in months or years, extreme hypergammaglobulinemia, viral persistence and death due to glomerulonephritis are additional characteristics. A lymphocyte blastogenesis assay has been utilized to examine the responsiveness of infected mink to viral associated antigens. Mink with advanced disease, regardless of genotype, respond strongly to viral associated antigens as well as to specific protein antigens to which they were sensitized prior to AD infection. Uninfected mink or mink with nonprogressive infections do not respond to the viral associated antigens. The responding cells are "T" cells indicating that T cell function is not abrogated in infected mink. However, if T cell function is compromised by neonatal thymectomy hypergammaglobulinemia and lesions typical of Aleutian disease do not develop. The relation of T cell unction to the massive uncontrolled B cell expansion that occurs in this lymphoproliferative disorder might be defined by studying such cellular phenomenon.

Lymphocyte blastogenesis. To examine cellular function in Aleutian disease of mink, we have utilized a lymphocyte blastogenesis assay and have examined the response of lymph node cells from AD infected and normal mink to viral associated and specific antigens. Mink with elevated serum gamma globulin levels and high antibody titers respond strongly to viral associated antigens or to specific antigens (KLH or HGG) to which they were sensitized prior to ADV infection. Uninfected mink or mink that are viremic, but without significant elevation of antibody titer or increased serum gamma globulin levels do not respond to the viral associated antigens. The responding cells are nylon wool nonadherent cells (T cells).

Aleutian and nonAleutian mink infected with virulent viral isolates respond strongly to viral associated antigens but nonAleutian mink fail to respond if they are infected with a relatively avirulent isolate, even though they exhibit transient viremia. Thus, T cell responsiveness correlates with viral replication and established disease but appears to provide no protective or antiviral effect. The influence of neonatal thymectomy (T cell depletion) on Aleutian disease and immune function in mink was also studied. Lymph node cells from 7 of 32 neonatally thymectomized mink yielded stimulation indices at least 10-fold lower than appropriate controls when they were cultured with concanavalin A or phytohemagglutinin. The same mink also failed to generate a normal antibody response to the T dependent antigen, keyhole limpet hemocyanin. When subsequently infected with Aleutian disease, one of the animals failed to generate an antibody response to ADV and the serum gamma globulin level did not increase significantly. At death lesions consistent with Aleutian disease were not observed. Serum and selected tissues from this animal are being assayed for virus. If usual viral titers are realized it would seem that T cell function does not influence viral replication in mink but does relate to the antibody response to virus. Cellular interactions and regulatory mechanisms might then be defined.

Publications: None

EPIDEMIOLOGY BRANCH
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RESEARCH HIGHLIGHTS

The following sections summarize this year's developments in our research program and discuss their significance in relation to the epidemiology and ecology of the diseases under investigation. Some of these findings and others are bases for 32 publications. An additional 21 papers are scheduled for publication in scientific journals.

RICKETTSIOSES

Our collaborative studies have continued to be fruitful in providing us with field, microbiological and clinical materials from which we can obtain new information about rickettsiae and rickettsial diseases in this country and abroad. We have continued our participation as a World Health Organization Collaborating Centre for Rickettsial Reference and Research in which role we have provided reference reagents and expertise, particularly in tickborne rickettsial diseases and Q fever, to various domestic and foreign health agencies.

The thrust of our research effort has continued toward goals stated in last year's report. Rickettsial surveys of ixodid ticks that bite man or are involved in the natural maintenance of Rickettsia rickettsii in various areas of the United States are aimed at determining the variety and nature of spotted fever-group (SFG) rickettsiae, their relation to human infection, and their interaction with the causative agent of Rocky Mountain spotted fever (RMSF). It is now clear that the number of serotypes of SFG rickettsiae systemically infecting Dermacentor andersoni and D. variabilis, the proven vectors of RMSF, is limited to 4. Only one, R. rickettsii, has been etiologically definitely associated with human illness. R. rhipicephali is widely distributed in the United States in at least 4 species of ticks comprising 2 genera, yet it has never been found in the Northeast where RMSF occurs. R. montana is prevalent in at least 2 species of ticks in all regions surveyed east of the Rocky Mountains but it has never been identified on the West Coast. It increasingly seems likely that these 2 serotypes are stable variants from R. rickettsii. The fact that these organisms may be highly prevalent in foci where R. rickettsii is seldom found suggests natural interference in the transmission cycle of R. rickettsii. Preliminary experimental findings of Dr. Burgdorfer that these 2 organisms do indeed block establishment of R. rickettsii infection of ticks should help us to understand mechanisms pertinent to focality of RMSF and may suggest new approaches to control of this disease. Specific observations follow.

Virtually all of some 400 cases of RMSF occurring in Ravalli County, Montana, during the last 80 years have been acquired west of the Bitterroot River. The reason for this dichotomy has never been explained satisfactorily. Surveys of ticks from various localities on the east side have revealed a SFG rickettsia in ovaries of 50-75% of female *D. andersoni*. This agent is nonpathogenic for experimental animals but is transmitted ovarially to larval ticks of the next generation in which it causes generalized infection. Experimental attempts to superinfect female *D. andersoni* harboring this agent with *R. rickettsii* resulted in infection of all tissues except the ovary. *R. rickettsii* could not be transmitted to offspring. The precise mechanism for interference is still unclear. It appears that inability of *R. rickettsii* to invade ovarial tissues may be important in limiting distribution and maintenance of virulent rickettsiae in certain areas. Similar experimental studies demonstrated 100% interference between nonpathogenic strains of *R. montana* and *R. rhipicephali*, and virulent *R. rickettsii*. However, there was no interference between the nonpathogenic agent, 369-C—a SFG organism found in *D. andersoni* or *D. variabilis* throughout the U.S.—and *R. rickettsii*. The study to re-evaluate transmission of rickettsiae by tick sperm is nearly complete. While rickettsiae were demonstrated in developmental sperm cells, none could be observed in mature spermatozoa and all attempts to demonstrate rickettsiae in maturing oocytes and oviposited eggs were negative. This evidence suggests that transmission by tick sperm is not an important mechanism for natural maintenance of *R. rickettsii*. Selected cells derived from epithelial tissue of meadow voles supported persistent infection with *R. rickettsii* through 10 serial passages todate. This system may provide a useful experimental model for investigating biochemical and ultrastructural parameters of persistent infection characteristic of rickettsiae under natural conditions. A rapid method was developed by Dr. Todd whereby biopsies and other small specimens can be readied for electron microscopic (EM) examination within one hour after acquisition. The procedure has potential value for rapid laboratory diagnosis. In collaborative projects with outside agencies, Dr. Burgdorfer participated in the first phase of a 2-3 year study to determine the nature of rickettsial flora of man-biting ticks in Switzerland. Three species of ixodid ticks were collected in the state of Tessin. Nearly one-third of 437 *D. marginatus* were hemolymph test (HT)-positive for SFG rickettsiae. An organism serologically resembling *R. slovaka*, first described by Czech workers and quite similar to *R. sibirica*, was subsequently isolated from these ticks. Since *R. sibirica* has been etiologically implicated in North Asian tick typhus, possibilities that the *D. marginatus* rickettsia may be associated with tickborne illnesses in Switzerland will be investigated. Additional isolates of the "Swiss agent" (see last year's report) were obtained from *Ixodes ricinus* during this visit. No rickettsiae were detected in the few *Haemaphysalis punctata* collected. In a collaborative study with personnel of the Agricultural Experiment Station on ecology of *R. rickettsii* in Connecticut, the most significant serologic finding was the presence of fluorescent antibodies, apparently specific for *R. rickettsii*, in 39 of 48 raccoons tested. (Burgdorfer)

Studies in California have attempted to determine etiology and arthropod source of tick-associated RMSF-like illnesses that have occurred in recent years in coastal counties beyond the range of *D. andersoni*. Rickettsial surveys of the 3 most important man-biting species of ticks in these areas have demonstrated presence of 4 serotypes. Three, including *R. rhipicephali*, unclassified 364-D, and unclassified 369-C serotypes are present in *D. occidentalis*. Only 369-C-like organisms have thus far been found in *D. variabilis* and only Oregon, Tillamook-like strains have been detected in *I. pacificus*. 364-D is the most likely candidate among these rickettsiae as a cause of human disease. Strains of this serotype are antigenically similar but not identical to *R. rickettsii*. They were found in greatest abundance on premises where an RMSF case was acquired. In convalescent sera from 4 coastal patients with diagnostic antibody responses to SFG, 364-D isolates were more effective than other serotypes, including *R. rickettsii*, in reducing antibody titers by adsorption. The reverse was true in a Montana patient convalescing from classical RMSF. This finding suggests slight but significant antigenic differences in *D. occidentalis*, 364-D-like strains and classical strains of *R. rickettsii* from *D. andersoni*. Three rickettsial serotypes have been obtained from *H. leporispalustris* ticks taken from California jackrabbits. They include *R. rickettsii*-Hlp type, the unclassified 369-C agent, and an isolate that in preliminary testing appears to be *R. canada*. The latter finding, if confirmed by further study, is of particular interest. Only one strain of *R. canada*, a member of the typhus group, has ever been recovered despite intensive search. The prototype, #2638, was isolated in 1963 from *H. leporispalustris* collected from sentinel rabbits in Ontario, Canada. What appears to be a new serotype was obtained from a single specimen of *D. parumapertus* collected from a jackrabbit. This is of lesser interest in view of the fact that *D. parumapertus* neither bites man nor is important in natural maintenance of *R. rickettsii*. In a collaborative epidemiologic study of RMSF in high incidence areas, Cabarrus and Rowan Counties, North Carolina, 61 cases of RMSF were confirmed serologically by microimmunofluorescence. This is approximately 3 times the average number of cases reported there each year. A rickettsial survey of *D. variabilis* collected in these 2 counties showed low rates of rickettsia-like organisms in HT-positive ticks when compared to *D. variabilis* obtained in a county of low RMSF incidence (Granville). Only one isolation of *R. rickettsii* was obtained (Rowan County). *R. montana* was equally distributed among ticks taken from the 3 counties, but the high prevalence of HT-positive ticks in Granville was accounted for entirely by organisms of the 369-C serotype. (Philip, Casper)

The clinical report of Kimbrough, Ormsbee, Peacock *et al.*, several years ago of Q fever endocarditis has resulted in recognition of a flurry of additional cases in this country from 5 of which biopsy, surgical and serological specimens were referred to this laboratory for diagnosis and study. *Coxiella burnetii* was isolated from resected aortic valves from 2 patients and from biopsy of a liver granuloma from a third. The course of serological responses of these patients, all of whom have been treated and are still living, has been studied by 3 serological procedures and the biological characteristics of the etiological agents from 3 individuals are being compared to *C. burnetii* from primary Q fever. When we are properly equipped to do so, cryostat sections of patient material will be examined for possible immunopathology arising from antigen-antibody complex deposition. In the meantime, we are exploring use of animal models to clarify pathogenesis of chronic Q fever. During the year an outbreak of primary Q fever associated with an infected goat herd at Montana State University was investigated. (Peacock, Philip)

LEGIONNAIRES' DISEASE (LD)

Our studies of LD continue on a reduced scale. Our interests have been directed toward developing more satisfactory methods for serogrouping strains of LD agent. Present methods used by CDC are based on direct immunofluorescence using conjugates prepared from known strains with differing antigenic characteristics. Shortcomings of this approach perhaps can be eliminated by use of serotyping techniques established for study of chlamydiae and rickettsiae. Methods being examined at present include microagglutination and ELISA tests using guinea pig antisera and indirect microimmunofluorescence using mouse antisera. During these studies it was shown that formalin inactivation of LD bacillus was superior to heat inactivation for serologic purposes. In continuing studies initiated last year with Dr. Williams (LMSF) and Dr. Emilio Weiss (Naval Medical Research Institute) to explore metabolic requirements of growth of LDB, growth in a simple yeast extract broth medium was monitored by scanning electron microscopy (SEM) that showed appearance of many bud-like protusions during stationary growth phase coinciding with release of soluble LD group antigen in the medium. Hopefully, studies of dynamics of infection of primary chick embryo cell culture can be pursued during the coming year as well as possibilities for early diagnosis by detecting circulating LD agent antigen in buffy coats and mononuclear cells. (Peacock, Thomas)

SYSTEMATICS OF TICKS

Systematic studies of ticks have continued to focus particularly on those of potential medical importance and to use SEM for their description. SEM studies of the Haller's organ of argasid ticks of the genus *Argas* are nearly complete. Similar studies are planned for *Ornithodoros*. In continuing studies of the *O. capensis* complex, redescription of *O. amblus* and *O. muesebecki* is either complete or nearly so and this year we hope to turn attention to ticks closely related to *O. denmarki* from which many viruses have been recovered. Our manual utilizing SEM micrographs for identification of ixodid ticks of the genus *Dermacentor* is almost complete. Based on response to publication of a similar manual on *Ixodes* in the U.S., this new manual should also be well received by health workers in this country. Progress has also been made to clarify *Ixodes* species of the *angustus* complex in Spearfish Canyon, South Dakota, a focus of continuing activity of Powassan virus. At least 3 new species related to I. oldi from Africa were recognized and we are continuing our studies of medically and agriculturally important rhipicephalid species in Ethiopia and now also from other areas in Africa and the Middle East. To-date some 6 distinct morphological entities have been recognized. The RML tick data base-Smithsonian computer system is now operational. In April, 3 documents were derived from the RML data file in the computer including a list of the species of Ornithodoros of the world alphabetically by country, a list of hosts from which more than one species of tick are found concurrently, and the distribution of Dermacentor species in North and South America by country, state, county and host. We are awaiting a dedicated line to RML and investigating purchase of terminal, printer and asynchronymous communication. (Clifford, Keirans)

A preliminary draft of the book updating and redescribing the Nuttall collection of ticks, the world's third largest, deposited in the British Museum (Natural History) has been completed. Since many tick species described by Nuttall and coworkers are vectors of human and animal disease agents, a manuscript selecting lectotypes for 58 tick species in the Nuttall collection has also been prepared and submitted for publication. (Keirans)

Taxonomic studies have continued on parasitic mites of Surinam and Saudi Arabia. Resulting diagnostic keys should be particularly helpful to public health entomologists in the latter country. Study of psoric mange mites of laboratory primates has been expanded to clarify the confused taxonomy of this group. (Yunker)

ARTHROPOD-BORNE VIRUSES

In the project to determine occurrence and natural history of tick-borne disease agents, 56 viral, 33 rickettsial, 6 spiroplasmal and 14 bacterial isolates were obtained from 599 pools of ticks originating in 5 states. Viruses included Colorado tick fever, Powassan, Sapphire II, Six-gun City and 3 undescribed but previously recognized entities. Of particular interest was isolation of 3 strains of Powassan virus from I. spinipalpis ticks collected in the same locality in South Dakota where Powassan virus was obtained by Dr. Eklund in 1964. This finding indicates a stable focus of a disease agent identical or similar to the prototype strain recovered from a fatal illness in Canada some years ago. Two of the undescribed virus isolates were from Alaska and are members of the Kemerovo and Sakhalin serogroups. The third has been repeatedly isolated in Xenopus and tick cell lines from D. occidentalis collected in Oregon. The latter virus has been partially characterized. The virion contains RNA and a lipid-bound coat, is 100-220 nm in size and is sensitive to elevated temperatures and acid pH. Rickettsial isolates were SFG organisms belonging to R. montana, R. rhipicephali, and the unclassified I. pacificus and 369-C serotypes. Of interest here was the fact that the latter strains from H.

leporispalustris could not generally be propagated in Vero cell culture. On the other hand, isolation was readily obtained in Xenopus and several tick cell lines. In collaboration with Dr. Tully, LID, we isolated a new and unique spiroplasm from 6 pools of I. pacificus ticks collected in Oregon. Heretofore, spiroplasms have been recovered only from H. leporispalustris. Francisella tularense was also isolated in antibiotic-free Xenopus cells from many pools of H. leporispalustris collected from Alaskan lagomorphs. These strains gave rise to well-defined plaques, a new finding as regards this bacterium. (Yunker, Clifford, Thomas)

A most significant development in the project on vector-pathogen relationships was the establishment of 6 embryonic cell lines from the ixodid ticks, D. variabilis and D. parumapertus. Most of the effort in this project was spent in characterizing the lines, testing their susceptibility to various viral, rickettsial and spiroplasmal agents, and production of adequate stocks for distribution to interested investigators in this country and abroad. Cells from one line of D. variabilis, now in its 56th passage, are predominantly diploid and are comprised equally of male and female karyotypes. This line grows well at both 27°C and 37°C and monolayers incubated at the latter temperature can be split approximately every 5 days. Three other lines of D. variabilis are also fibroblastic but grow more slowly, while 2 lines derived from D. parumapertus, also predominantly fibroblastic, do not withstand higher temperatures. The spectra of infectious agent susceptibility of these lines are being investigated. Preliminary observations indicate broad host susceptibility to arboviruses when compared to more selective mosquito cell lines. A new virus from D. occidentalis collected in Oregon grew abundantly in the tick lines as well as Xenopus cells whereas it could not be cultivated in mammalian cells. Growth was demonstrated also for several kinds of rickettsiae and the spiroplasm, suckling mouse cataract agent. Use of tick lines for cultivation of Anaplasma marginale is being investigated in collaboration with Oklahoma State University investigators. Future studies will continue to be concerned with in vitro tick-cell/pathogen relationship with special emphasis on application of arthropod cell lines to the problems of vector-borne diseases. (Yunker)

PATHOGENESIS OF SLOW VIRAL DISEASES

This project continues to wind down and no new experiments have been initiated in 2 years. Nevertheless, additional important observations on the relation of spongiform encephalopathy of sheep, viz. scrapie, and Creutzfeldt-Jakob disease (CJD) of man, have been obtained. So far, 3 goats inoculated intracerebrally with brain tissue from 3 patients with fatal CJD have become affected with clinical manifestations and neurohistologic changes characteristic of caprine scrapie. Signs of illness first appeared in 2 African pygmy goats 43 months after inoculation and in one Nubian crossbred goat at 37 months. The former 2 goats were sacrificed at 8 and 24 weeks. The latter goat is still being observed 6 weeks after onset of clinical manifestations. Five other goats receiving 2 of the positive specimens appear to be normal after nearly 5 years. Three other groups of 2 goats each show no signs of illness after inoculation of CJD material 3 years ago and 4 groups of 2 goats each seem to be normal 4 1/2 years after inoculation. Study conditions were such that disease in the 3 affected goats almost certainly did not arise from inadvertent exposure to scrapie virus. The findings show not only that putative CJD virus in human brain can cause encephalopathy in a natural host of scrapie, but also that the disease it causes is indistinguishable from scrapie. They raise important questions as regards etiologic identity and epidemiology of the 2 diseases. In the coming year, the remaining goats will be observed for neurologic disease and a backlog of histologic material will be evaluated. (Hadlow)

IMMUNOBIOLOGY OF RELAPSING FEVER

A new project was initiated this year to elucidate pathogenesis of relapsing fever and dynamics of seroconversion of Borrelia during course of infection. Little is known about mechanisms of clinical relapse and means by which the relapsing fever organism undergoes seroconversion and changes in surface antigens thereby escaping immunologic counterreaction by the host. On basis of published reports, louse-borne type of Borrelia consists of 2 serotypes and the tickborne type, 4 or possibly 5 serotypes. In attempts to obtain pure populations of serotypes and with use of mice for preparation of antisera and as experimental hosts, it was shown that 2 or 3 relapses involving 7 different serotypes that could be readily distinguished by staining with fluoresceinated antiserum were obtained after inoculation of single organisms. When as many as 25 spirochetes/mouse representing each of the 7 serotypes were used to initiate infection, 24 serologically distinct subpopulations were subsequently detected. The 24 serotypes were then used to initiate infection and the following observations were made. Relapses occurred when circulating populations reached levels $\geq 10^5$ organisms/ml. The blood was not sterile between relapses. Each relapse population generally consisted of 2 or more serotypes. Change from one serotype to another between relapses is usually, but not always, gradual. Future studies will be directed toward elucidating dynamics of seroconversion. (Stoenner)

Annual Report
Epidemiology Branch
Rocky Mountain Laboratories
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National Institute of Allergy and Infectious Diseases
October 1, 1979 to September 30, 1980

ADMINISTRATIVE REPORT

Currently, the Epidemiology Branch (EB) comprises 3 sections: Arthropod-Borne Diseases, Epidemiology, and Histopathology. The EB is manned by 8 Ph.D.-level scientists, including 4 in the Arthropod-Borne Diseases Section, 3 in Epidemiology, and one in Histopathology. In addition, there are 11 supporting staff and one secretary assigned to the Office of the Chief. A permanent Chief of EB has not yet been appointed. The total complement is 20. All scientists are tenured personnel.

In addition, there are 2 scientists assigned to the Arthropod-Borne Diseases Section under IPA contract. Dr. William Todd, Colorado State University, has completed his first of 2 years in Dr. Burgdorfer's laboratory and is providing much needed expertise in defining vector-pathogen relationships by electron microscopy. Dr. Beverly R. Norment, Mississippi State University, arrived in June for one year to conduct studies on the susceptibility of dogs and rodents to infection by rickettsiae representing various serotypes of the spotted fever group.

The EB continues to provide consultative expertise and rickettsial antigens and antisera to qualified foreign and domestic investigators in keeping with its role as WHO Collaborating Centre for Rickettsial Reference and Research.

In September 1980 an NIAID-sponsored Conference on Rickettsiology and Rickettsial Diseases was held at RML. Approximately 80 rickettsiologists principally from the United States attended to hear 52 scientific papers and exchange new scientific information about their respective areas of interest.

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HONORS AND AWARDS

The following activities reflect recognition of EB by their peers in the scientific community.

Editorial Boards of Journals:

Dr. W. Burgdorfer - Acta Tropica
Dr. C. E. Yunker - Journal of Parasitology

Drs. Burgdorfer, Yunker, Clifford, Keirans, Hadlow and Philip reviewed manuscripts for J. Med. Entomol., Econ. Entomol., J. Parasitol., Ann. Entomol. Soc. Am., The Auk, Am. J. Trop. Med. Hyg., Infect. Immun., Vet. Pathol., Am. Coll. Lab. Animal Med., CRC Press, Bull. Entomol. Soc. Am.

Professional Posts:

Dr. R. N. Philip - Secretary, Committee pro tem to organize an American Society for Rickettsiology and Rickettsial Diseases

Dr. W. Burgdorfer - Member of the Research and Thesis Committee of the Zoological Institute, University of Neuchâtel, Switzerland; Co-Chairman, Organization Committee, Rickettsiology Conference, RML, Sept. 1980

Invited Lectures and Participation in Meetings and Symposia

Dr. W. Burgdorfer was invited by Zoological Institute (Prof. Dr. A. Aeschlimann, Director) of the University of Neuchâtel, to participate in a tick/pathogen study in the state of Tessin, Switzerland.

Dr. C. E. Yunker was invited to participate in the 2nd International Conference on the Impact of Viral Diseases on the Development of African and Middle Eastern Countries, Nairobi, Kenya.

Other Honors:

Dr. W. J. Hadlow was awarded the Distinguished Alumnus Award from Ohio State University College of Veterinary Science, June 1980.

Mr. Dan Corwin received an invitation to serve as a research fellow at the Veterinary Research Institute, Onderstepoort, South Africa.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00061-18 EB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Natural History of Tick-Borne Rickettsial Agents and Their Public Health Significance

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. Burgdorfer	Res. Entomologist (Med.)	EB NIAID
OTHER:	R. N. Philip	Medical Director	EB NIAID
	L. A. Thomas	Research Microbiologist	EB NIAID
	E. A. Casper	Nurse Director	EB NIAID
	J. C. Williams	Biochemist	LMSF NIAID

COOPERATING UNITS (if any) Dr. J. N. MacCormack, CDC Branch, Dept. Human Resources, North Carolina; Drs. J. Anderson and L. Magnarelli, Conn. Agric. Res. Exp. Station; Dr. A. Aeschlimann, University of Neuchâtel, Switzerland.

LAB/BRANCH
Epidemiology Branch (RML), Hamilton, MT 59840

SECTION
Arthropod-Borne Diseases Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 5.5	PROFESSIONAL: 1.1	OTHER: 4.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is concerned with studies of Rocky Mountain spotted fever and other tick-borne rickettsial diseases in the United States and in other countries regarding ecology, identification, and characterization of the rickettsial agent(s) with their associated vectors. With collaboration of outside agencies (state health departments, public health laboratories, hospitals, physicians, etc.) source material for comparative studies on the various rickettsial types and information about the distribution of vector species associated with spotted fever in the United States is obtained. This project also considers the cellular and subcellular aspects of interaction between tick-borne agents, particularly rickettsiae, and their vectors.

Project Description:

Rocky Mountain spotted fever continues to be a significant health problem, and many outside agencies (state health laboratories, university research departments, hospitals, physicians) solicited our cooperation in their efforts to determine the epidemiologic, ecologic, and health impacts on the public. However, because our research program has shifted to studies of the biology of rickettsiae, particularly of the relationship(s) of the spotted fever group (SFG) agents to their tick vectors, our assistance to other agencies, as in the previous year, was limited to guidance and/or supplying diagnostic reagents. To study in the laboratory the biochemical and antigenic relationships of nonvirulent SFG agents to virulent rickettsiae (R. rickettsii in the U.S., R. conorii and R. sibirica in European countries), we continued our cooperation in a tick/rickettsial survey in North Carolina, and participated in a similar survey in certain parts of Switzerland.

Research accomplishments during the past year include the following findings:

Tick/rickettsial surveys on the Bitterroot Valley's east side, where human cases of spotted fever have never been recorded, revealed 50-75% of Dermacentor andersoni females infected with a SFG agent (East side agent) whose growth is limited to the ovarian tissues, the distal diverticula of midgut, and occasionally also to the malpighian tubules. The "East side agent" is morphologically indistinguishable from other SFG rickettsiae and is nonpathogenic for laboratory animals. It is ovarially transmitted to larval ticks where it produces a generalized infection including the tissues of the salivary glands, but in nymphs and adults, it exhibits a strong organotropism for the female genital system, the midgut in males and females, and occasionally also for certain parts of the malpighian tubules; hemocytes and other tissues are free of rickettsiae. Attempts to infect with virulent R. rickettsii tick females harboring the "East side agent" resulted in infection with R. rickettsii in all the tissues except the ovary. Consequently, there was no transovarial passage of R. rickettsii. Although the mechanism(s) of this interference between the "East side agent" and R. rickettsii is still unclear, it appears that inability of R. rickettsii to invade ovarial tissues represents an important factor limiting the distribution and maintenance of virulent spotted fever rickettsiae in certain areas.

In Connecticut where human spotted fever is rare (only 15 cases reported since 1965), ecologic investigations provided evidence of widely separated foci of SFG rickettsiae in inland and coastal areas. Rickettsia-like organisms were detected in 116 of 3,331 D. variabilis and in 2 of 513 Ixodes dammini. So far, all rickettsial isolates from ticks are of the R. montana serotype. A strain of R. rickettsii, on the other hand, has been recovered from the blood of a patient. Serologic surveys detected SFG antibodies in 22 of 38 gray squirrels, 48 of 192 raccoons, 31 of 659 white-footed mice, 14 of 549 white-tailed deer, 1 of 10 woodchucks, and 1 Eastern woodrat. Of the 48 seropositive raccoons, 39 had significant microimmunofluorescent titers against R. rickettsii, the others were positive against R. montana and the 369-C agent.

Following an invitation by the University of Neuchâtel, Switzerland, the PI of this project assisted in the organization of, and participated in, a field expedition into the mountainous parts of the state of Tessin (Switzerland) where goat industry provides an important livelihood of a typical rural human population. Most people as a result of close association with goats are frequently bitten by ticks, and dermatoses (with and without rash) of unknown etiology are said to occur every year. The first phase of the 2 to 3-year study was conducted in March through May 1980 and consisted in a tick/rickettsial survey throughout the area. Two species of ixodid ticks, Ixodes ricinus and D. marginatus, were found on vegetation as well as on goats. A third species, Haemaphysalis punctata, was collected off vegetation only. One hundred and forty-two (32.5%) of 437 D. marginatus and 58 (16%) of 363 I. ricinus were found to be infected with rickettsiae of the SFG. None of 28 H. punctata was positive. Indirect immunofluorescent tests identified the rickettsia in D. marginatus as R. slovaca, and the rickettsia in I. ricinus as the "Swiss agent" - a recently identified new SFG rickettsia associated with I. ricinus in other parts of Switzerland (see last year's report). Failure to isolate R. slovaca from I. ricinus or the "Swiss agent" from D. marginatus provides a typical example of vector specificity. Experiments to confirm this phenomenon are in progress. Purified antigens have been prepared from both species of rickettsia and have been forwarded to Neuchâtel for the serologic evaluation of sera from tick hosts as well as from humans.

There is 100% interference between the nonpathogenic rickettsial serotypes, R. montana and R. rhipicephali, and the virulent serotype, R. rickettsii. Ticks infected with the former serotypes cannot be infected with the spotted fever agent. The mechanism of this phenomenon is currently under investigation. It may explain scarcity of strains of R. rickettsii in areas where the nonpathogenic serotypes are abundant. There is no interference between the nonpathogenic agent 369-C - a rickettsialike microorganism found in ticks (D. andersoni and D. variabilis) throughout the U.S. - and R. rickettsii.

Re-evaluation of sexual transmission of rickettsiae by tick sperms is nearing completion. It appears that rickettsiae are incorporated into undifferentiated spermatogonial cells, early spermatocytes, and primary spermatocytes. However, rickettsiae have not as yet been observed in mature spermatozoa, and all attempts to demonstrate rickettsiae in maturing oocytes and in oviposited eggs were negative. This suggests elimination or loss of viability of the rickettsiae during spermatogenesis and questions the claim that "sexual transmission" of rickettsiae by tick sperms is a significant means for maintenance and distribution of these microorganisms in nature.

Selected cells derived from epithelial tissues of meadow voles (*Microtus pennsylvanicus*) were found to support persistent infection with rickettsiae including R. rickettsii. The balance between rickettsial growth and host-cell multiplication so far has been maintained for 10 passages during a 5-month period. To our knowledge, this is the only example of persistent infection by an obligate intracellular bacterium in cell culture. It provides the possibilities to investigate the biochemical and ultrastructural parameters of relationships between host tissues and parasites that may cause persistent infections.

A rapid method for embedding biopsies and other small specimens (tick tissues) for examination by electron microscopy was developed. Fixation, dehydration, infiltration and curing of the block are accomplished within 1 hr from acquisition of the sample. This method basically consists of the reaction of cyanoacrylate with vinyl cyclohexane dioxide which results in the formation of a crystal-clear 3-dimensional polymer. This processing of specimens provides an opportunity for rapid identification of arthropod-borne agents in vector tissues and increases the potential use of electron microscopy by diagnostic laboratories.

Publications:

Aeschlimann, A., Burgdorfer, W., Matile, H., Peter, O., and Wyler, R.: Aspects nouveaux du rôle de vecteur joué par *Ixodes ricinus* L. en Suisse. Note préliminaire. Acta Trop. 36: 181-191, 1979.

Magnarelli, L. A., Anderson, J. F., and Burgdorfer, W.: Rocky Mountain spotted fever in Connecticut: human cases, spotted fever-group rickettsiae in ticks, and antibodies in mammals. Am. J. Epidemiol. 110: 148-155, 1979.

Burgdorfer, W.: Current status of tick-borne typhus (Rocky Mountain spotted fever) in the United States. In *Proceedings of the 4th International Congress of Acarology, 1974*. Budapest, Akademiai Kaido, Publishing House of the Hungarian Academy of Sciences, 1979, pp. 355-359.

Linnemann, C. C., Jr., Schaeffer, A. E., Burgdorfer, W., Hutchinson, L., and Philip, R. N.: Rocky Mountain spotted fever in Clermont County, Ohio. II. Distribution of population and infected ticks in an endemic area. *Am. J. Epidemiol.* 111: 31-36, 1980.

Burgdorfer, W., Aeschlimann, A., Peter, O., Hayes, S. F., and Philip, R. N.: *Ixodes ricinus*: vector of a hitherto undescribed spotted fever group agent in Switzerland. Preliminary note. *Acta Trop*. 36: 357-367, 1979.

Hayes, S. F., Burgdorfer, W., and Aeschlimann, A.: Sexual transmission of spotted fever group rickettsiae by infected male ticks. I. Detection of rickettsiae in immature spermatozoa of *Ixodes ricinus*. *Infect. Immun.* 27: 638-642, 1980.

Bell, J. F.: Tularemia. In Steele, J. H. (Ed.): *CRC Handbook Series in Zoonoses*. Boca Raton, Florida, CRC Press, Inc., 1980, vol. II, pp. 161-193.

Burgdorfer, W.: The spotted fever-group diseases. In Steele, J. H. (Ed.): *CRC Handbook Series in Zoonoses*. Boca Raton, Florida, CRC Press, Inc., 1980, vol. II, pp. 279-301.

Bell, J. F. and Moore, G. J.: Allergic encephalitis, rabies antibodies, and the blood/brain barrier. *J. Lab. Clin. Med*. 95: 5-11, 1979.

Bell, J. F., Stewart, S. J., and Wikel, S. K.: Resistance to tick-borne *Francisella tularensis* by tick-sensitized rabbits: allergic klendusity. *Am. J. Trop. Med. Hyg*. 28: 876-880, 1979.

Nelson, W. A., Bell, J. F., and Stewart, S. J.: *Polyplax serrata*: cutaneous cytologic relations in mice that do (CFW strain) and do not (C57BL strain) develop resistance. *Exp. Parasitol*. 48: 259-264, 1979.

In press:

Burgdorfer, W.: Rickettsioses. In Hubbert, McCulloch and Schnurrenberger (Eds.): Chapter 34. Supplementum to "Diseases Transmitted from Animals to Man".

Burgdorfer, W., Cooney, J. C., Mavros, A. J., Jellison, W. L., and Maser, C.: The role of cottontail rabbits (*Sylvilagus* spp.) in the ecology of *Rickettsia rickettsii* in the United States. *Am. J. Trop. Med. Hyg*.

Feng, W. C., Murray, E. S., Burgdorfer, W., Spielman, J. M., Rosenberg, G., Spickert, C., and Waner, J.: Spotted fever group rickettsiae in *Dermacentor variabilis* from Cape Cod, Massachusetts. *Am. J. Trop. Med. Hyg*.

Burgdorfer, W.: Borreliae. In Johnson, R. C. (Ed.): *Manual of Clinical Microbiology*, 3rd ed.

Burgdorfer, W.: The arthropod vector as a selective transport system of animal and human pathogens. In *Comparative Aspects of Animal and Plant Pathogens*. Rockefeller Foundation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00063-10 EB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Immune Responses to Rickettsial Infections and Legionnaires' Bacterium

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. N. Philip	Medical Director	EB NIAID
	M. G. Peacock	Microbiologist	EB NIAID
OTHER:	E. A. Casper	Nurse Director	EB NIAID
	T. F. McCaul	Expert	LMSF NIAID
	L. A. Thomas	Research Microbiologist	EB NIAID
	J. C. Williams	Biochemist	LMSF NIAID

COOPERATING UNITS (if any) Dr. M. H. Smith, Vet. Research Lab., MSU, Bozeman, MT.

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TOTAL MANYEARS: 1.9	PROFESSIONAL: 0.5	OTHER: 1.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to study immune responses in man and experimental animals to natural and experimental rickettsial infections. Currently, we are studying chronic Q fever in man. Studies have involved isolation and characterization of Coxiella burnetii isolates from heart valves and the characterization of the immune response in chronic Q fever. We are also investigating the immune responses in man and experimental animals to natural and experimental infections of Legionella pneumophila, the causative agent of Legionnaires' disease (LD). This past year we compared suitability of various antigen preparations in the microimmunofluorescence test (micro-IF) and initiated a study of serological typing of strains of L. pneumophila using mouse antiserum. We also devised an ELISA test for measuring antibody responses in LD patients and to experimental infection in guinea pigs.

Project Description:

Q fever. Primary Q fever usually subsides within 2 or 3 weeks. However, chronic disease occasionally supervenes 3 to 20 years after primary infection. The features of chronic Q fever are usually endocarditis, liver dysfunction and granulomata, vegetation and mineralization of heart valves, and occasionally, glomerulonephritis from immune-complex deposition.

During the past 2 years, we have studied 6 patients with chronic Q fever from the United States. Isolates of *C. burnetii*, phase I, were made from 2 of 3 resected aortic heart valves with vegetations. Direct immunofluorescent staining confirmed *C. burnetii* in biopsy sections from a liver granuloma in a fourth patient. Diagnosis was established serologically in all 6 patients. High levels of humoral phase I antibody were determined by complement-fixation (CF), microagglutination (MA) and microimmunofluorescence (micro-IF) tests. Elevated IgM, as well as IgG, levels appear to be characteristic of Q fever endocarditis. Radial immunodiffusion tests indicated that all patients had high serum IgM and IgG levels but normal concentrations of IgA immunoglobulin. The high titered phase I and II antibodies were predominantly IgG. The 2 *C. burnetii* isolates from valvular vegetations grew abundantly in embryonated hens' eggs. Purified antigen yields were 3 to 5 times greater than with any other strains in our collection.

In collaboration with Dr. Smith, we investigated an outbreak of abortion in goats and consequent *C. burnetii* infection of Veterinary Research Laboratory personnel. Direct FA staining of impression smears of placental cotyledons from 2 aborted goat fetuses revealed massive infection by *C. burnetii*. Inoculation of placental tissue induced antibodies specific for *C. burnetii* in guinea pigs, and abundant growth of *C. burnetii* in yolk sac after second egg passage. MA, CF and micro-IF tests showed that 5 of 59 individuals associated with the diseased herd had ≥4-fold rise in antibody titer to *C. burnetii* by one or more tests. Seven other individuals had significant levels of antibody in single sera suggestive of recent infection.

We plan to study cryostat sections of human heart valves from chronic Q fever patients with specific immunological fluorescent reagents in attempts to visualize *C. burnetii*, and to determine if complexed antigen-antibody is present. We are also attempting to develop an experimental laboratory animal model for chronic Q fever. The best candidates for such a model appear to be guinea pigs or sheep since *C. burnetii* can be isolated from placental tissues of these animals several years after primary infection. In man, colonization of heart valves by *C. burnetii* is often superimposed on previous valvular damage resulting from recurrent rheumatic fever or congenital anomalies. Valvular damage can be induced by chemical means in laboratory animals. Injected killed rickettsia are found in liver and spleen for long periods after inoculation. The role of tissue deposition of immune complexes or in potentiating pathological changes in chronic Q fever will also be studied experimentally.

Legionnaires' disease. Prototypes of Legionnaires' disease bacterium (LDB) representing the 4 known serogroups were obtained from the Center for Disease Control (CDC) including Knoxville I, serogroup 1; Togus I, serogroup 2; Bloomington 2, serogroup 3; and Los Angeles I, serogroup 4. Other isolates in our collection include Philadelphia 2, serogroup 1, Philadelphia S, serogroup 1, and Bellingham I, serogroup 1. We isolated another strain in guinea pigs and embryonated eggs in 1974 from cord clot and placental tissues from a healthy young woman at delivery of a normal infant in Frederick, Maryland. The isolate, Frederick I, is LDB according to DNA homology determined at CDC and is considered to be serogroup 4.

Preliminary observations with these 8 LDB strains indicate that convalescent guinea pig serum can be used to serotype strains of LDB by MA and micro-IF. Philadelphia 2 and Philadelphia S, both of which had been isolated during the Philadelphia outbreak, could not be distinguished from each other. However, pronounced antigenic differences were discovered between them and Bellingham I and Frederick I, and between Bellingham I and Frederick I. These strains could not be differentiated on basis of antibody reactions with convalescent human serum in the micro-IF test. We are evaluating the ELISA test for measuring antibody responses in the guinea pig model, and micro-IF cross-tests of mouse antisera as an additional means for serogrouping isolates. We also found that formalin-inactivated LDB antigen is more reactive and strain-specific than heat-inactivated antigen that had been used by CDC.

Our initial finding that LDB can utilize glucose for energy prompted us to further study growth of LDB in a liquid medium incorporating graded concentrations of glucose and glutamine. We confirmed the report of Ristroph and coworkers that LDB can be grown aerobically in a simple yeast extract broth medium. Growth of Philadelphia 2 strain of LDB in the yeast extract broth medium has been followed for 110 hours by measuring turbidity and colony forming units at 4 hr intervals. Morphological and cytological changes of the bacterium during growth were monitored by scanning electron microscopy (SEM). LDB exhibited changes in size and formed many bud-like protusions during the stationary growth stage. Appearance of protusions coincided with appearance of soluble LDB group antigen in growth medium.

All LDB strains tested produce plaques in primary chick embryo cell culture (PCETC). Direct FA staining shows that LDB can invade and multiply within cells. Plaque size, morphology, time of appearance and cytoplasmic growth resemble those of typhus group rickettsiae. Additional study of LDB infection of PCETC cells is planned. PCETC cells inoculated with LDB will be monitored by SEM at various time periods after infection.

Early diagnosis of Legionnaires' disease is desirable. A study is proposed in which buffy coat from LDB-infected guinea pig whole blood will be examined by direct FA for LDB. If negative, remaining cells will be used to initiate primary monocyte culture. Cultures will be examined for LDB-infected cells after incubation.

Publications:

Kimbrough, R. C., Ormsbee, R. A., Peacock, M., Rogers, W. R., Bennetts, R. W., Raaf, J., Krause, A., and Gardner, C.: Q fever endocarditis in the United States. Ann. Intern. Med. 91: 400-402, 1979.

In press:

Ormsbee, R. A., Peacock, M. G., and Fiset, P.: Differences in infectivity and pathogenicity among three strains of the agent of Legionnaires' disease. J. Clin. Microbiol.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00065-07 LMSF EB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Antigens and Classification of the Rickettsiae (see also page 28-7 of Annual Report of Laboratory of Microbial Structure and Function)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	R. L. Anacker	Research Microbiologist	LMSF NIAID
OTHER:	E. A. Casper	Nurse Director	EB NIAID
	W. Burgdorfer	Res. Entomologist (Med.)	EB NIAID

COOPERATING UNITS (if any) Dr. R. S. Lane, University of California, Berkeley; Dr. J. N. MacCormack, North Carolina Division of Health Services, Raleigh, NC.

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Epidemiology Branch & Laboratory of Microbial Structure & Function (RML)

SECTION

Epidemiology Section and Rickettsial Diseases Section

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TOTAL MANYEARS: 1.8	PROFESSIONAL: 0.9	OTHER: 0.8

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long-range objectives of this project are to develop practicable procedures for classification of spotted fever- and typhus-group rickettsiae and to determine the nature and biological properties of rickettsial antigens and constituents. This part of the project focuses on differentiating serologically the kinds of rickettsiae found in arthropod vectors known to transmit rickettsial disease agents to man or are involved in the maintenance of pathogenic rickettsiae in nature with particular reference to the United States. The microimmunofluorescence (micro-IF) test is an excellent screening test for this purpose. Ultimately, representative strains of the important serotypes will be systematically examined by more refined techniques for antigenic and other biologic characteristics (see also 28-7).

Project Description:

1. The 1979 collaborative study of spotted fever group (SFG) rickettsiae with the California Department of Health (Lane) has been completed and the results are being compiled for publication. The purpose of the study was to identify the kinds of SFG rickettsiae to be found in the man-biting ticks from coastal areas of California including *Dermacentor occidentalis*, *Ixodes pacificus*, and *D. variabilis*. Since 1954, 14 of the 25 serologically confirmed indigenous cases of Rocky Mountain spotted fever (RMSF) have occurred in coastal areas of California well beyond the western limits of *D. andersoni*, the only proven vector of RMSF in western United States. A total of 85 isolates of SFG rickettsiae were obtained from 877 adult ticks obtained by flagging vegetation in 4 coastal areas ranging from Humboldt County in the north to San Diego County in the south. Seventy-nine of these typed as *R. rhipicephali*; 3 as the unclassified 364-D agent closely related to *R. rickettsii*; 2 as the unclassified 369-C organism; one similar to the unclassified *I. pacificus* rickettsia widely distributed in coastal Oregon. The first 2 serotypes were found only in *D. occidentalis*; the 369-C-like serotypes only in *D. variabilis*. Indirect evidence based on antibody-absorption tests of convalescent sera suggested that at least some of the RMSF cases from coastal California are etiologically associated with the 364-D-like strains but not the *R. rhipicephali* or the unclassified *I. pacificus* serotypes (see Project No. *00081-19* for details).

2. In collaboration with Dr. Burgdorfer's unit and the North Carolina Division of Health Services (MacCormack) 72 SFG isolates were obtained from hemolymph test (HT)-positive *D. variabilis* obtained at 17 collecting sites in 3 Piedmont counties of North Carolina. Two of the counties (Rowan and Cabarrus) are areas of high RMSF incidence and are participating in an epidemiologic study of RMSF (see Project No. *00081-19*); the other (Granville), is in an area of low incidence. Prevalence of HT-positive ticks was higher in Granville County than the other 2 counties. Sixty-five isolates were obtained from Granville County; 59 typed as 369-C and 6 as *R. montana*. Seven isolates were obtained from Rowan and Cabarrus Counties. However, none of these was 369-C, but 6 were *R. montana* and one was *R. rickettsii*. The significance of this unusual distribution of serotypes is at present speculative.

3. The California studies were resumed this year a) to obtain a more complete understanding of the distribution of serotypes in the state particularly in *D. variabilis* and in areas where *D. occidentalis* and *D. andersoni* overlap, b) to determine the predominant serotypes on premises where RMSF was acquired, c) and to identify the kinds of rickettsiae to be found in *Haemaphysalis leporispalustris* collected from *Lepus californicus*.

The most significant preliminary findings are as follows:

a) The only serotype thus far found in D. variabilis is 369-C.

b) No isolates were obtained from northeastern counties where D. occidentalis and D. andersoni overlap.

c) A higher prevalence of 364-D-like organisms was observed in D. occidentalis collected on the premises where a RMSF case occurred in 1979 than in this species obtained in other sites in California.

d) Nearly one-half of the 191 H. leporispalustris collected from 5 jack-rabbits were HT-positive for rickettsia-like organisms. Only 2 isolates were obtained in Vero cells from the 93 HT-positive ticks infected with small short rods. Both typed as 369-C. However, additional attempts by Jack Cory to recover strains of this organism in tick- and Xenopus-cell culture were successful even when isolation attempts in Vero cells were negative. Of particular interest was the isolation of an organism from one of 7 H. leporispalustris infected with typical rickettsia-like coccobacilli that in preliminary micro-IF tests appears to be a strain of R. canada, a member of the typhus group. If this is verified, this will be only second strain of R. canada ever recovered and will indicate that the isolation of the R. canada prototype (#2678) from H. leporispalustris collected in 1963 in Ontario, Canada, from the other side of the continent was not just an oddity.

Future course of this project will continue along present lines. As far as the California studies are concerned, more information is needed about the possible vector relationship of D. variabilis to RMSF. Direct evidence of the nature of the causative agent awaits isolation of rickettsiae from untreated illnesses in acute stages. This winter, we hope to begin to characterize representatives of the 364-D serotype and to determine the degree of antigenic relationship to classical strains of R. rickettsii. Representative isolates of 369-C from various arthropod hosts will be similarly examined. The presumptive R. canada isolate will be examined for various biologic similarities to the prototype. Hopefully, DNA homology and EM morphology studies will be conducted on the 369-C and R. canada isolates.

Publications:

In press:

Philip, R. N. and Casper, E. A.: Serotypes of spotted fever group rickettsiae isolated from Dermacentor andersoni (Stiles) ticks in western Montana. Am. J. Trop. Med. Hyg.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AI 00069-49 EB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Systematics and Vector Relationships of Certain Parasitic Arthropods

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. M. Clifford	Scientist Director	EB NIAID
OTHER:	J. E. Keirans	Res. Entomologist (Med.)	EB NIAID
	C. E. Yunker	Scientist Director	EB NIAID

COOPERATING UNITS (if any) Drs. H. Hoogstraal, NAMRU-3; D. Sonenshine and P. Homsher, Old Dominion Univ.; P. Teel, Texas A&M; D. Stiller, USDA; F. S. Lukoschus, Catholic Univ., Netherlands; Miss Jane Walker, Div. of Vet. Services, Onderstepoort.

LAB/BRANCH
Epidemiology Branch (RML), Hamilton, MT 59840

SECTION
Arthropod-Borne Diseases Section

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 4.1	PROFESSIONAL: 1.8	OTHER: 2.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The activities of this project currently comprise four main functions: 1) Identification of ticks received from various individuals and government agencies throughout the world. Only one other institution in the world is capable of performing this service. 2) Systematic study of certain groups of parasitic arthropods. The foremost tool in systematic studies, the scanning electron microscope, has greatly aided in elucidating taxonomic concepts in acarines actually or potentially involved in transmission of disease agents. 3) Retrieval and use of tick data in the Smithsonian data retrieval system. Installation of a mini-computer-word processor system at RML to communicate with NIAID and Smithsonian computers. 4) Colonization of medically important arthropod vectors, which are furnished to outside investigators.

PHS-6040
(Rev. 10-76)

Project Description:

The primary object of this project is the systematic treatment, including taxonomy and classification, biology, ecology and colonization of parasitic arthropods of known or potential medical importance.

Argasid ticks. Our studies on the Haller's organ of ticks of the genus Argas are nearly completed. We are comparing our results with some recently published studies by Russian workers that dealt mainly with species that occur in Russia. After the studies on Argas are completed, we plan to investigate the same structures in the genus Ornithodoros.

Our studies on the Ornithodoros capensis complex continued: The redescription of O. amblus is published and we are making progress on a redescription of O. muesebecki. In the future we plan to clarify the status of the ticks that are closely related to O. denmarki. We have isolated numerous viruses from this group of ticks and their status is uncertain because of the difficulty in identifying species related to O. denmarki.

Ixodid ticks. Our manual that makes extensive use of scanning electron microscope pictures to help identify ticks of the genus Dermacentor is nearly completed.

Significant progress was also made on clarification of an Ixodes species of the angustus complex that occurs in Spearfish Canyon. We made a 4-day field trip to the canyon and collected various stages in the life cycle. These ticks have been reared and we are now ready to provide descriptions of all active stages.

An extensive study of several species in Africa related to Ixodes oldi has been completed. Three and possibly four new species have been recognized and manuscripts describing them will be prepared as time allows.

Considerable effort was expended on our continuing studies of medically and agriculturally important rhipicephalid species in Africa. As suggested last year, we have been collaborating with Mr. Rupert Pegram on the R. sanguineus complex in Ethiopia. To date over six distinct morphological entities have been recognized. We are now expanding these studies to include other areas of Africa and the Middle East. Two large studies are underway on collections from Iraq and Mali.

Mite studies. Work on the Macronyssidae of Surinam and the parasitic fauna of Saudi Arabia continued. Resulting publications will provide faunal lists and diagnostic keys for public health entomologists. A major collection of macronyssids from Washington and Oregon was identified. Our study of psoric mange mites of laboratory primates has been expanded in order to clarify the confused taxonomy of this group. By request, identification of parasites were made for numerous outside institutions or specimens and keys were made available to them. Institutions requesting help included: Florida Dept. of Agriculture and Consumer Services, Royal Ontario Museum, Alaska Dept. of Fish and Game, Univ. of Wisconsin, Univ. of Montana, Old Dominion University, Ohio State Univ., and the Blair Research Laboratory, Salisbury, Rhodesia.

RML-Smithsonian computer data. In April 1980, Drs. Clifford and Keirans worked for one week at the computer center of the National Museum of Natural History. They produced three documents from the RML tick data file in the Smithsonian computer: 1) A list of the species of Ornithodoros of the world alphabetically by country (233 p.); 2) A list of hosts in which more than one species of tick are found concurrently (2 volumes: 614 p.); and 3) the distribution of the Dermacentor species of North and South America by county, state, county and host (145 p.). These listings proved that the RML data base and the Smithsonian computer system are fully functional for the first time and the entire system is now operational. We are currently awaiting a dedicated line to RML and investigating the purchase of terminal, printer and asynchronyous communication. The data base at the National Museum of Natural History is also being entered into the computer at NIH.

Nuttall Collection Updating of all host information, geographical data, including locality coordinates, collectors and dates for the 3,972 Nuttall tick collections has just been completed. A preliminary draft of the book on this, the third largest tick collection in the world, has also been completed. Because many of the tick species described by Nuttall and his coworkers are vectors of human and animal disease agents, it is important to stabilize the nomenclature relating to these species. For this reason, a manuscript selecting lectotypes for 58 tick species in the Nuttall collection has recently been prepared and submitted.

Service-oriented activities. The RML insectary continues to provide excellent support to both RML scientists, visiting investigators, and researchers in other parts of the country. In 1979-1980 we provided on 68 separate occasions 87,574 ticks of 10 different species. Identification of thousands of tick specimens as a service function by this unit continues to provide us with numerous specimens for our research needs.

The future course of the project will emphasize the use of the SEM to clarify taxonomic problems among various genera of ticks. Special attention will be devoted to the groups of ticks from which we are isolating viruses and other disease agents. We will also stress the work on groups of ticks like the African rhipicephalids that are proven vectors of diseases of humans and other animals. We plan to produce additional practical guides to the identification of ticks that are amply illustrated with SEM photos.

Publications:

Hoogstraal, H., Clifford, C. M., and Keirans, J. E.: The *Ornithodoros* (*Alectorobius*) *capensis* group (Acarina: Ixodoidea: Argasidae) of the Palearctic and Oriental region. *O.* (*A.*) *coniceps* identity, bird and mammal hosts, virus infections, and distribution in Europe, Africa, and Asia. *J. Parasitol.* 65: 395-407, 1979.

Corwin, D., Clifford, C. M., and Keirans, J. E.: An improved method for cleaning and preparing ticks for examination with the scanning electron microscope. *J. Med. Entomol.* 16: 352-353, 1979.

Clifford, C. M., Hoogstraal, H., Radovsky, F. J., Stiller, D., and Keirans, J. E.: *Ornithodoros* (*Alectorobius*) *amblus* (Acarina: Ixodoidea: Argasidae): Identity, marine bird and human hosts, virus infections, and distribution in Peru. *J. Parasitol.* 66: 312-323, 1980.

In press:

Hoogstraal, H., Clifford, C. M., Keirans, J. E., and Wassef, H. Y.: Recent developments in biomedical knowledge of *Argas* ticks (Ixodoidea: Argasidae). *Proc. 5th Intl. Congress of Acarology*

Keirans, J. E.: The tick collection (Acarina: Ixodoidea) of the Hon. Nathaniel Charles Rothschild deposited in the Nuttall and general collections of the British Museum (Natural History). *Bull. Brit. Mus. Nat. Hist. (Zool.)*

Stark, H. E., Inlao, I., Clifford, C. M., Keirans, J. E., and Lakshana, P.: A preliminary checklist of the ticks of Thailand. *Appl. Sci. Res. Corp J. Thailand*

Keirans, J. E., Clifford, C. M., and Yunker, C. E.: Rocky Mountain Laboratory reference collection of ticks and parasitic mites (Acari). *A Guide to the Parasite Collections of the World*

Keirans, J. E., Clifford, C. M., and Hoogstraal, H.: Identity of the nymphs and adults of the Galapagos iguanid lizard parasites, *Ornithodoros* (*Alectorobius*) *darwini* and *O.* (*A.*) *galapagensis* (Ixodoidea: Argasidae). *J. Med. Entomol.*

Yunker, C. E., Binninger, C. E., Keirans, J. E., Beecham, J., and Schlegel, M.: Clinical mange and other parasitic infestations of black bears in Idaho. *J. Wildl. Dis.*

Fain, A. and Yunker, C. E.: Lutracarus canadensis, n. g., n. sp. (Acari, Listrophoridae) from the otter, Lutra canadensis (Schreber). J. Med. Entomol.

Yunker, C. E. and Radovsky, F. J.: Parasitic mites of Surinam: XXXVI. A new genus and two new species of Neotropical Macronyssidae (Acari, Mesostigmata). J. Med. Entomol.

Homsher, P. J. and Yunker, C. E.: Bilateral gynandromorphism in Dermacentor andersoni (Acarina: Ixodidae): morphologic and cytogenetic analysis. J. Med. Entomol.

Appendix 1. PL-480 Project 03-063N. Epidemiological and Biological Aspects of Tick-Borne Infections in Egypt and Elsewhere, NAMRU-3, Cairo, Egypt

Annual Funding: $123,000

During our visit we conferred with all three of the major investigators concerned with the project at NAMRU-3, Agouza Virus Laboratory and Cairo University.

NAMRU-3: The members of the Bibliography Section have continued their high level of productivity. During the past year they reviewed over 800 reprints. They are rapidly approaching completion of the Annotated Bibliography of Tickborne Viruses which will consist of over 1000 pages. Many references for volume 6 of the author index have been assembled and this tome should be published in 1980. This group provided considerable support in preparation of a monographic review on The Epidemiology of Tickborne Crimean-Congo hemorrhagic fever in Asia, Europe and Africa which recently appeared as a special 110-page issue of the Journal of Medical Entomology.

This unit also translated 85 articles, mostly from Russian, and numerous ticks were illustrated by the staff artist for manuscripts in preparation.

Agouza Virus Lab: Considerable new information resulted from the serological studies being conducted at this unit. For example, in a serological study to determine the presence of Thogoto virus in Egypt it was shown that this agent undoubtedly infects humans, domestic animals and commensal rats. This virus is deemed important because Thogoto virus was isolated from humans in Nigeria with serious optic neuritis and a fatal meningoencephalitis. In other studies on Bhanja and Rift Valley fever virus they discovered that the prevalence of antibodies in sera tested was significantly higher if the HI test was employed rather than the CF test. They intend to study the comparative dynamics of CF and HI antibodies in mice experimentally infected with Rift Valley fever virus.

Cairo University: As we stated in our 1978 report, this part of the project would suffer if the amendment requesting additional equipment were not granted. Without this additional equipment the staff at Cairo University was unable to obtain the results with the virological aspects of this project. Instead they have attempted to do some basic studies on a bacteria they isolated from *Hyalomma dromedarii*. During the coming year they intend to expand their studies on this agent.

This unit also initiated several studies on the serological and physiological properties of argasid ticks. These investigations included a study of pathenogenesis in *O. savignyi*, mating behaviour of *O. savignyi* and *H. impeltatum*, and the effects of various hosts on development and reproduction of these same two tick species.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00080-13 EB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Tick-Borne Disease Agents

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. E. Yunker	Scientist Director	EB NIAID
OTHER:	C. M. Clifford	Scientist Director	EB NIAID
	J. E. Keirans	Res. Entomologist (Med.)	EB NIAID
	R. N. Philip	Medical Director	EB NIAID
	L. A. Thomas	Research Microbiologist	EB NIAID

COOPERATING UNITS (if any) Dr. J. Casals, YARU, New Haven; Dr. R. Gresbrink, Oregon State Bd. Hlth., Portland; Dr. H. Hoogstraal, NAMRU-3, Cairo, Egypt; Dr. B. Easterday, Dept. of Vet. Sci., Univ. Wisconsin, Madison; Dr. E. Easton, S. Dakota State Univ.

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SECTION
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TOTAL MANYEARS: 2.3	PROFESSIONAL: 1.0	OTHER: 1.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In this project the incidence, distribution and ecology of tick-borne disease agents, arboviruses and rickettsiae, are studied. These pathogens are isolated in tissue cultures or animals from field-collected materials and are serologically identified. Viruses, if new, are physicochemically and biologically characterized and taxonomically classified. Of particular importance is the delineation of vector-relationships and human disease potential of the increasing number of tick-borne viruses in the United States.

Project Description:

This project is concerned with documenting the occurrence and natural history of tickborne disease agents. Mosquitoborne arboviruses are relatively well-studied in comparison to those that are tickborne and this project helps to fill an obvious void in the study of vectorborne disease. This year we tested over 2,700 ticks in 599 pools. Samples originating in Alaska, Arkansas, California, Oregon and South Dakota yielded 56 viral, 33 rickettsial, 6 spiroplasmal and 14 bacterial isolates.

Viruses. Viruses isolated included Colorado tick fever, Powassan, Sapphire II, Sixgun City and three undescribed, but previously recognized, entities. Three Powassan virus isolates were from *Ixodes spinipalpis* ticks collected in the Black Hills of South Dakota. RML had previously recorded this virus from the same tick and locality in 1964. Two of the undescribed viruses, both from Alaska, are members of the Kemerovo and Sakhalin serogroups. The third is an undescribed agent which we have repeatedly isolated in *Xenopus* and tick cell lines from *Dermacentor occidentalis* ticks in western Oregon (see last report). Extensive efforts to physicochemically, biologically, and electron microscopically characterize the latter virus have been hampered by its low titer and poor pathogenicity for laboratory animals. However, we have determined that the virion contains RNA and a lipid-bound coat, is between 100-220 nm in size and is sensitive to elevated temperatures as well as to acid pH. Further studies are in progress.

Rickettsias. The 33 rickettsial isolates were all made in *Xenopus*, tick or Vero cells and were identified as members of the spotted fever group, specifically *Rickettsia montana*, *R. rhipicephali* and the Quarry strain of a unique rickettsia previously seen from Oregon. One rickettsia was repeatedly isolated from California populations of *Haemaphysalis leporispalustris* (Hlp) ticks. Although the organism could be observed in hemocyte smears, it could not be isolated in homeothermic cell cultures. An immune mouse serum prepared from *Xenopus* cells infected with this rickettsia was tested by microimmunofluorescence against 29 rickettsial antigens, revealing relationships with spotted fever organisms. The rickettsia is being further studied by Dr. Philip, RML.

Spiroplasms. In collaboration with Dr. J. Tully, LID, we investigated the association of spiroplasms with tick hosts other than *H. leporispalustris*. No spiroplasms have heretofore been isolated from any tick species other than Hlp. Nearly all ticks tested this year were routinely filtered and inoculated into spiroplasma isolation medium, after which they were sent to LID for observation. This resulted in the recognition of a new and unique spiroplasm from 6 pools of *Ixodes pacificus* ticks collected in Oregon.

Bacteria. Thirteen isolations of *Francisella tularense* were made from as many pools of Hlp ticks collected from Alaskan lagomorphs. The isolations were made by the plaque technique in antibiotic-free *Xenopus laevis* cells incubated at 34°C. The bacterial plaques, all well-defined and clear, appeared unexpectedly during the course of tests for viruses or rickettsiae. To our knowledge, the tissue culture plaque technic has not previously been used for tularemia diagnosis.

Upon request of the Yale University Medical School, we tested in poikilothermic cell cultures tick material for pathogenic agents. Three types of cultures, including tick cell lines, yielded only bacterial organisms, which we presume to be contaminations from the ticks. However, the bacteria were sent to Yale for further study. We honored additional requests for our services, reagents, or for technical training made by public health, veterinary and wildlife disease investigators in 7 states of the United States as well as in Germany and France.

The isolation of Powassan virus in the Black Hills of S. Dakota after 16 years confirms that this locality is a persistent and stable enzootic focus. Powassan encephalitis is a rare, but sometimes fatal, human infection. The S. Dakota locality is frequented by vacationers and those with outdoor occupations. Further studies to define the natural cycle of this virus are warranted. The new viral, spiroplasmal and rickettsial agents isolated from man-biting ticks in coastal Oregon should be considered as candidates in the causality of Lyme disease, recently recorded from the West Coast of the United States.

Publications:

Yunker, C. E., Clifford, C. M., Keirans, J. E., Thomas, L. A., and Rice, R.C.A.: Aransas Bay virus, a new arbovirus of the Upolu serogroup from *Ornithodoros capensis* (Acari: Argasidae) in coastal Texas. *J. Med. Entomol.* 16: 453-460, 1979.

Clifford, C. M.: Tick-borne viruses of seabirds. In Kurstak, E. (Ed.): *Arctic and Tropical Arboviruses*. New York, Academic Press, Inc., 1979, pp. 83-100.

Yunker, C. E., Thomas, L. A., and Cory, J.: Phenetic relationships of viruses of the Hughes serological group. In Kurstak, E. (Ed.): *Arctic and Tropical Arboviruses*. New York, Academic Press, Inc., 1979, pp. 211-229.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00081-19 EB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
The Epidemiology of Human Infections of Special Interest

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. N. Philip	Medical Director	EB NIAID
OTHER:	E. A. Casper	Nurse Director	EB NIAID
	L. A. Thomas	Research Microbiologist	EB NIAID
	W. Burgdorfer	Res. Entomologist (Med.)	EB NIAID

COOPERATING UNITS (if any) Dr. R. Emmons, Viral and Rickettsial Disease Lab., Calif. Dept. of Health, Berkeley; Dr. Catharine Wilfert, Duke Univ. School of Medicine, Durham, NC; Dr. A. Steere, Yale Univ. School of Med., New Haven, CT.

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SECTION
Epidemiology Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.7	1.2	0.5

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☒ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project emphasizes epidemiologic investigations of infectious diseases of particular interest to the research program of this laboratory. Current investigations include studies of the etiologic relationship of various spotted fever-group serotypes to human illness, study of epidemiologic attributes pertinent to occurrence of Rocky Mountain spotted fever (RMSF) in a high incidence area, and improvement in laboratory methods for diagnosis of rickettsial infections, particularly RMSF.

PHS-6040
(Rev. 10-76)

Project No. Z01 AI 00081-19 EB

Project Description:

Etiologic role of spotted fever group (SFG) agents to human infection. Collaboration has continued with Dr. Emmons in attempts to determine the etiology of spotted fever-like illnesses acquired in coastal counties of California. Four patients from western counties north of San Francisco were confirmed as having RMSF by serodiagnostic antibody responses. As is usual with human convalescent sera, the predominant antibodies were broadly reactive against most SFG rickettsiae. Therefore, the antigenic nature of the etiologic agent could not be identified on basis of type-specific reactions. However, indirect evidence as to etiology was obtained by cross-absorption microimmunofluorescence (micro-IF) tests. Absorption efficiency of classical strains of *R. rickettsii* of Montana origin were compared with *R. rickettsii*-like isolates from California (364-D prototype) in tests of sera from a Montana patient with laboratory-acquired RMSF (R-strain origin) and from the 4 California cases. Classical *R. rickettsii* was 2 to 4 times more efficient than California isolates in removing antibody in convalescent serum from the Montana case of RMSF. Conversely, California 364-D-like isolates were 2-4 times more effective than *R. rickettsii* in removing SFG antibody from California cases. The California *Ixodes pacificus* and *R. rhipicephali* isolates from California (see Project No. 00065-07 LMSF EB) were ineffective in removing serum antibody from all California patients as well as from the Montana patient with RMSF. These findings suggest that the causative rickettsiae of at least some cases of RMSF in coastal California are slightly different from *R. rickettsii* and raise the question as to whether an efficient spotted fever vaccine against classical RMSF would be prophylactically useful against the California disease.

Epidemiology of RMSF in North Carolina. In collaboration with Drs. Burgdorfer (EB), Anacker (LMSF) Wilfert (Duke University) and the North Carolina Division of Health Services we are participating in a 3-year study of RMSF in North Carolina with the purpose of defining epidemiologic attributes pertinent to occurrence of disease in a high incidence area. The Piedmont counties of Rowan and Cabarrus have consistently had among the highest reported attack rates of RMSF in the nation, about 1 per 10,000 per year. Our role is to obtain information on the relationship of enzootic SFG serotypes to tick-associated illnesses and to provide serologic support (micro-IF test) for the study. Cooperation of health officials in the participating counties is excellent and most spotted fever-like illnesses are brought to the attention of study personnel for clinical, epidemiologic and laboratory investigation. In 1979, 61 cases of RMSF in the 2 counties were serologically confirmed by micro-IF. This number is roughly 3-fold higher than the average number of cases reported for the past 5 years and indicates underestimate of the actual incidence of disease from normal reporting channels in a state that is acutely aware of the spotted fever problem. With one exception, all convalescent sera reacted as specifically or more so to *R. rickettsii* than to other members of the SFG. One patient with typical disease did develop antibodies that reacted primarily against R. rhipicephali. In attempts to determine frequencies of past infection testing of Red Cross sera by donor-age cohort is underway. Preliminary observations on persistence of antibody suggest that serologic procedures will have limited value as methods for retrospective determination of the true incidence of infection. Of interest is the fact that pre-antibiotic-era patients with RMSF usually had higher residual levels of antibody to R. rickettsii than those who acquired disease after introduction of broad-spectrum antibiotics.

Enzyme-linked immunosorbent assay (ELISA) and RMSF. A considerable effort was directed toward developing and evaluating an ELISA for serodiagnosis of RMSF and particularly as a means for detecting antigen in urine during acute-preeruptive stages of disease. A serodiagnostic test was worked out and shown to be reliable and sensitive when compared to established serologic procedures. However, there appear to be no particular advantages of ELISA over other recently-developed tests being increasingly used for serodiagnosis of RMSF. Our attempts to use ELISA for detection of antigen have been disappointing. Urine samples collected by Duke University collaborators during early stages of RMSF were examined by a "double-sandwich" procedure with negative or nonspecific results. High dilutions of coating and indicator antibody must be used to eliminate nonspecific binding of test components. In such high dilutions, the kinds of antibody corresponding to the antigen being excreted are perhaps diluted beyond the endpoint of reactivity. For the moment at least, we have discontinued our efforts to detect antigen by ELISA.

Other. In collaboration with Dr. Steere. we tested several groups of coded control as well as acute and convalescent sera from patients with Lyme disease, by micro-IF against the battery of rickettsial serotypes in our collection. Lyme disease is a syndrome of unknown etiology in New England thought to be associated with the tick, Ixodes dammini. Although sera from some patients had low antibody titers against several SFG agents, no significant association of reactivity with illness could be demonstrated. At present, there is no indication for rickettsial etiology of Lyme disease.

This project will continue to investigate the etiology of spotted-fever-like illness in California. Direct evidence of the nature of the infecting SFG rickettsia will be sought by attempts to isolate the organism during acute stages of untreated disease. Our serologic support of the North Carolina epidemiologic study will continue for at least one more year. In collaboration with Dr. Burgdorfer, we plan to serotype isolates of rickettsiae from North Carolina patients with RMSF.

Publications:

Philip, R. N., Paretsky, D., Weiss, E., and Wisseman, C. L., Jr.: Rickettsiology Conference. J. Infect. Dis. 141: 112-118, 1980.

Philip, R. N.: Scrub typhus. In Steele, J. H. (Ed.): CRC Handbook Series in Zoonoses. Boca Raton, Florida, CRC Press, Inc., 1980, vol. II, pp. 303-315.

Philip, R. N.: Typhus-group rickettsiae. In Steele, J. H. (Ed.): CRC Handbook Series in Zoonoses. Boca Raton, Florida, CRC Press, Inc., 1980, Vol. II, pp. 317-335.

In press:

Hechemy, K. E., Anacker, R. L., Philip, R. N., Kleeman, K. T., Sasowski, S. J., and Michaelson, E. E.: Detection of Rocky Mountain spotted fever antibodies by a latex agglutination test. J. Clin. Microbiol.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00082-19 EB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Relation of Viruses to the Genesis of Chronic Disease.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. J. Hadlow	Res. Veterinarian (Path.)	EB NIAID
OTHER:	None		

COOPERATING UNITS (if any) Dr. S. B. Prusiner, Dept. of Neurology, University of California, San Francisco.

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SECTION
Histopathology Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is mainly concerned with characterizing several naturally occurring slow viral diseases of domestic animals that resemble important, etiologically obscure diseases of man. Two diseases receive most attention: scrapie of sheep and goats and Aleutian disease of mink. Their clinicopathologic, virologic, and immunologic features are studied to provide an understanding of their pathogenesis and natural history. Information from such studies will help define the unusual host-virus relationships that result in protracted disease in man and animals.

Project Description:

This project is designed to learn more about the unusual host-virus relationships that result in slowly evolving diseases after long incubation periods. The main diseases studied are scrapie of sheep and goats and Aleutian disease of mink. Although widely different diseases, each is an excellent model for manipulation in the laboratory. Scrapie is the prototype of the socalled viral spongiform encephalopathies, whereas Aleutian disease is a chronic glomerulopathy resulting from the damaging effects of infectious immune complexes. The primary objective of the research is to study the disease process in each infection. Most attention is directed to obtaining information on the pathogenesis and natural history of each disease.

New experimental work was discontinued 2 years ago. So the project is now devoted to making observations on those experiments that have been underway since then. The lack of supporting laboratory studies has made this a less than satisfactory endeavor.

As mentioned in last year's report, the only part of this project yielding new observations has to do with goats inoculated intracerebrally with brain tissue from persons dying of Creutzfeldt-Jakob disease (CJD). So far, 3 goats have become affected with a disease clinically indistinguishable from caprine scrapie. Each had received a different inoculum. Two goats, African pygmies, began showing signs about 43 months after inoculation. One was killed 8 weeks later, and the other 24 weeks later. Both had neurohistologic changes characteristic of caprine scrapie. The third goat, a Nubian crossbred, became affected 37 months after inoculation. As in the other 2, onset of the disease was dominated by scratching and rubbing the skin with horns, muzzle, and hind feet. Now, about 6 weeks after onset, the goat appears depressed and is beginning to have an uncertain, stilted gait. It continues to eat, but is losing weight. Two other goats that had received the same inoculum still seem normal.

Three other groups of 2 goats each will have been inoculated 3 years in August of this year. So far, all seem normal. Four groups of 2 goats each have been inoculated 4 1/2 years, and they too seem normal. Five goats remaining in the first 2 positive groups will have been inoculated 5 years in September of this year. Although several were thought to be in the prodromal stage of the encephalopathy a year ago, all seem normal now.

Conditions of this study have been such that the disease in the 3 goats almost certainly did not arise from inadvertent exposure to scrapie virus. Thus, the findings reported here show not only that the putative CJD virus in human brain can cause encephalopathy in a natural host of scrapie; but also that the disease it causes is indistinguishable from scrapie. Two interpretations of this conclusion are apparent: either CJD virus and scrapie virus are the same, or essentially so as other observations suggest, or they are different and scrapie is less a specific etiologic entity than it is a clinicopathologic one. If these viruses are in fact the same, the findings reported here, however suggestive they may be, do not establish this. Given the present technical limitations for distinguishing the two viruses from one another, the extent of their similarity, or indeed their common identity, will not be known until both are purified and their chemical structures determined.

On the other hand, if these viruses are different, then the nosologic status of scrapie as a natural disease of sheep and goats is brought into question. As has been true historically for many diseases when their causes are not well enough understood to allow an etiologic definition, scrapie is probably best thought of now as a distinctive clinicopathologic entity. That is, it is a recognizable combination of clinical signs accompanying characteristic, though qualitatively nonspecific, neurohistologic changes. If this definition is accepted, then virus from such diverse sources as human brain (as described here) and mink brain (as described in earlier annual reports) can be said to have caused scrapie, at least in the goat. All this has a bearing on the epidemiology of both scrapie and CJD.

Whether the findings reported here are exceptional or represent what might happen often is not known. Continued observation of the goats in the other groups may help clarify this. Meanwhile, the possible pathogenicity of scrapie virus for man and, more specifically, its relation to the occurrence of CJD remain uncertain.

In the coming year, then, the remaining goats will be watched for evidence of neurologic disease. And some mink will be followed for the late appearance of scrapie or Aleutian disease. Most of the time will be spent evaluating the large amount of experimental data collected during the active phase of this project. This will be facilitated when clerical help is provided. In addition, much histologic material remains to be evaluated. Peripheral to the main project but of great comparative interest are the observations being made on neoplasia in mink. Undoubtedly, the specimens at Rocky Mountain Laboratory represent the largest collection of neoplasms from mink in the world. They will be dealt with as time permits.

Publications:

Hadlow, W. J.: Myopathy associated with congenital hydrocephalus in Hereford calves. In Andrews, E. J., Ward, B. C., and Altman, N. H. (Eds.): Spontaneous Animal Models of Human Disease. New York, Academic Press, Inc., 1979, pp. 102-103.

Hadlow, W. J., Race, R. E., Kennedy, R. C., and Eklund, C. M.: Natural infection of sheep with scrapie virus. In Prusiner, S. B. and Hadlow, W. J. (Eds.): Slow Transmissible Diseases of the Nervous System. New York, Academic Press, Inc., 1979, vol. 2, pp. 3-12.

Prusiner, S. B., Garfin, D. E., Baringer, J. R., Cochran, S. P., Hadlow, W. J., Race, R. E., and Eklund, C. M.: On the partial purification and apparent hydrophobicity of the scrapie agent. In Prusiner, S. B. and Hadlow, W. J. (Eds.): Slow Transmissible Diseases of the Nervous System. New York, Academic Press, Inc., 1979, vol. 2, pp. 425-463.

Hadlow, W. J., Kennedy, R. C., Race, R. E., and Eklund, C. M.: Virologic and neurohistologic findings in dairy goats affected with natural scrapie. Vet. Pathol. 17: 187-199, 1980.

Hadlow, W. J., Cheville, N. F., and Jellison, W. L.: Occurrence of pox in a northern fur seal on the Pribilof Islands in 1951. J. Wildl. Dis. 16: 305-312, 1980.

In press:

Hadlow, W. J. and Ward, J. K.: Atresia of the right atrioventricular orifice in an Arabian foal. Vet. Pathol.

Hadlow, W. J., Prusiner, S. B., Kennedy, R. C., and Race, R. E.: Brain tissue from persons dying of Creutzfeldt-Jakob disease causes scrapie-like encephalopathy in goats. Ann. Neurol.

Hadlow, W. J.: Animal models of human neurologic disease. Am. J. Pathol. suppl., D. B. Hackel (Ed.).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00084-11 EB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Vector-Pathogen Relationships

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	C. E. Yunker	Scientist Director	EB NIAID
OTHER:	L. A. Thomas	Research Microbiologist	EB NIAID

COOPERATING UNITS (if any) Dr. Kouka Abdel-Wahab, Al Azhar University, Cairo, Egypt; Dr. Mamdouh Y. Kamel, National Research Center, Cairo, Egypt; Dr. D. Knudson, Yale University, New Haven, CT; Dr. K. Kocan, Oklahoma State University, Stillwater.

LAB/BRANCH
Epidemiology Branch (RML), Hamilton, MT 59840

SECTION
Arthropod-Borne Diseases Section

INSTITUTE AND LOCATION
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project deals with the interactions of vector-borne microorganisms, arboviruses, rickettsiae and protozoans, and cells of haematophagus arthropods, mainly ticks. Model in vitro systems are developed and applied to studies of pathogen host-range, growth dynamics, persistence and interference. After prolonged growth in these systems, pathogens are examined for changes in invasiveness, virulence, or antigenic characteristics. By contract with collaborative laboratories overseas, we are studying critical factors influencing survival of ticks and of pathogens within ticks from a biochemical, histochemical, and physiological standpoint.

PHS-6040
(Rev. 10-76)

Project Description:

Major emphasis was placed on the production of adequate stocks of our six, recently developed, embryonic cell lines from ixodid ticks and their characterization with regard to karyology, growth dynamics and virus-supporting abilities. Cells of one line from *Dermacentor variabilis*, which is now in its 56th passage, are predominately diploid and comprised of approximately equal numbers of male and female karyotypes. The cell number doubles every 182 hours at 27°C, but growth rate increases significantly (population doubling time = 70 hr) at an incubation temperature of 37°C. Cells are mostly fibroblastic and form semi-confluent monolayers that may be split at 5-day intervals. Three other *D. variabilis* lines are fibroblastic, but grow somewhat more slowly at 37°C, averaging 7-10 days between passages. Two lines of *D. parumapertus* cells also are predominately fibroblastic but do not withstand the higher temperatures of incubation. Here, the female karyotype predominates, and numerous aneuploid cells are seen. One line, now in its 27th passage, is subcultured weekly, while the other is passed every 3 weeks. All six lines are extremely sensitive to the presence of toxic substances in the growth medium. Unlike mosquito cells, which are relatively selective with regard to arbovirus infection, the tick cells support a wide variety of arboviruses, which replicate without perceptible cytopathic effect, or production of plaques. Both mosquitoborne and tickborne members of the family Togaviridae (Chikungunya, O'nyong-nyong, St. Louis encephalitis, yellow fever, Langat, Powassan) replicated to higher levels in one or more of these lines, whereas, a non-vectorborne Togavirus, Modoc, did not. The tickborne viruses, Colorado tick fever and Kemerovo (Reoviridae) and Sawgrass (unclassified), also grew in these cells. A new virus, which we recently isolated from *D. occidentalis* ticks collected in Oregon, grew in the tick cells. This virus had repeatedly been recovered in a poikilothermic cell line (*Xenopus*) but could not be isolated in conventional homeothermic systems (Vero cells, newborn mice). When frozen tick triturates that had originally yielded the virus were thawed and retested in *D. variabilis* cells, isolations were readily made as revealed by specific immunofluorescence reactions and plaque assay in *Xenopus* cells. Upon request, some of the new tick cell lines were distributed to interested investigators in the U.S., Germany, China and U.S.S.R.

These tick cell lines were also tested for their ability to support growth of tickborne microorganisms other than viruses. Two different rickettsial species of the spotted fever group grew to higher levels in *D. variabilis* cells, which cultures were then used to prepare potent and specific immunofluorescence antigens.

The growth in tick cell cultures of a spiroplasm, SMCA (suckling mouse cataract agent), was assayed in comparison with its growth in other cell systems, in its standard growth medium (SP-4) and in cell-free cell culture media. Growth of SMCA in tick, mosquito and *Xenopus* cells, as well as in all media, was evident. The tick cells gave the highest yield and the Xenopus cells, the lowest. However, SMCA growth in the former was not much higher than in the standard medium, while growth in cell-free Xenopus cell culture medium was significantly higher than in Xenopus cells.

A collaborative project with Oklahoma State University on use of arthropod cell cultures for growth of Anaplasma marginale was initiated. Organisms resembling anaplasms were identified in infected tick guts by both Gimenez and Hoechst (DNA) stains. This material was inoculated into various arthropod cell systems. Results are pending.

As in the past years, we trained visiting scientists and technicians, both domestic and foreign, in tissue culture and virological methodology and provided them and others with cultures, viruses, serological reagents and instructional materials.

Future studies will be concerned with the in vitro tick-cell/pathogen relationship with special emphasis to be placed on applications of the system to problems in vectorborne disease. The establishment of tick cells in continuous culture has proven a difficult task. No other cell lines from Dermacentor ticks are available. Our cell lines should find use in various aspects of biomedical research, including isolation of temperature sensitive pathogens, latency, development of antigens and vaccines and control of both vectors and the pathogens they transmit.

Publications:

Bhat, U.K.M. and Yunker, C. E.: Susceptibility of a tick cell line (Dermacentor parumapertus Neumann) to infection with arboviruses. In Kurstak, E. (Ed.): Arctic and Tropical Arboviruses. New York, Academic Press, Inc., 1979, pp. 263-275.

Yunker, C. E. and Meibos, H.: Culture of embryonic tick cells (Acari: Ixodidae). TCA Manual 5: 1015-1017, 1979.

Yunker, C. E. and Cory, J.: Plaque assay method for arboviruses in mosquito cells. TCA Manual 5: 1077-1080, 1979.

Anderson, J. F., Magnarelli, L. A., and Kurz, J.: Intraerythrocytic parasites in rodent populations of Connecticut: Babesia and Grahamella species. J. Parasitol. 65: 599-604, 1979.

In press:

Yunker, C. E., Cory, J., and Meibos, H.: Continuous cell lines from embryonic tissues of ticks (Acari: Ixodidae). In Vitro.

Appendix 1. PL-480 Project 03-030-N. In vitro and in vivo Studies of Certain Tickborne Viruses in Vector and Host Cells, Al Azhar University, Cairo, Egypt.

Annual Funding: $110,444

This project investigates the contrasting behavior of a tickborne virus, Quaranfil (QRF), which is infectious for man in Egypt, in vector- as opposed to vertebrate-host tissues. Research areas currently under study include vertical transmission of virus in vector and non-vector ticks, the role of temperature sensitive virus variants in vector efficiency, and nucleic acid synthesis and polypeptide turnover in infected host cells. The agreement has been extended through April 1982, without additional funding, to permit completion of projects and investigation of additional research areas suggested by current results.

Appendix 2. PL-480 Project 03-051-N. Biochemistry and Physiology of Tick Vectors of Disease.

Annual Funding: $57,404

This project provides basic knowledge concerning the chemical and physical environment in which tickborne pathogens exist when not infecting the vertebrate host. Areas investigated during the second year of the project were: Nucleic acids of Dermacentor andersoni and Argas arboreus, Lipovittelins of Hyalomma dromedarii, purines and enzyme kinetics of ticks, and histology of embryonic development. These data aid understanding of factors influencing pathogen survival and development in tick vectors of disease, and provide analytical data useful in the formulation of tick tissue culture media.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AI 00200-01 EB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Immunobiology of Relapsing Fever

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: H. G. Stoenner Asst. Sci. Director, NIAID (RML)
OTHER: None

COOPERATING UNITS (if any) None

LAB/BRANCH
Office of the Scientific Director, NIAID

SECTION

INSTITUTE AND LOCATION
NIAID, NIH, Bethesda, MD 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is principally concerned with elucidating the pathogenesis of relapsing fever and the dynamics of seroconversion of the organism.

PHS-6040
(Rev. 10-76)

Project Description:

Although more than a century has elapsed since Obermeier discovered that *Borrelia* caused relapsing fever in man, little is known about the relapse mechanism or the means by which the organism undergoes seroconversion. It is generally accepted that the organisms change their surface antigens and thereby escape the immunologic reaction of the host, but little is known about the interaction between the host and the organism. Some contend the organism has a fixed spontaneous "mutation" rate and that relapses are caused by changed populations that emerge. Other investigators contend the change is induced by antibody. On the basis of published reports, the louse-borne type consists of 2 serotypes and the tick-borne type has 4, possibly 5 serotypes. Many investigators assume that the population of a given relapse consisted of a single pure serotype. As will be shown later, the assumption was not valid and it has led to a great deal of confusion about relapsing fever.

Before any investigation of seroconversion could be initiated, it was necessary to obtain pure populations of serotypes and specific fluoresceinated antiserum against each serotype. Initially the relapses from 6 mice were studied and specific antiserums were prepared against each relapse population. These mice, inoculated with a single organism, had 2 or 3 relapses involving 7 different serotypes that were readily distinguished from each other by staining with fluoresceinated antiserum. Groups of mice were then inoculated with 25 organisms/mouse of each of the 7 serotypes and the population of each relapse was characterized. Inocula used to initiate infections with these serotypes had less than 1% contamination with heterologous serotypes. By this process, 24 serologically distinct populations were identified and specific fluoresceinated antisera were prepared against serotype. Infections initiated with each of the 24 serotypes were then studied. The following observations were made: 1. It is generally thought that the blood stream of an infected animal (or man) is free of *Borrelia* between relapses. The blood always contained *Borrelia* until mice had experienced infection with a sufficient number of serotypes to establish complete immunity. "Relapses" occur when *Borrelia* populations reach concentrations of 10^5/ml or more; between relapses, the population may subside to less than 800/ml, but the organism can always be detected by blind passage in normal mice. 2. Relapse populations rarely consisted of a single serotype. Most relapse populations had at least 2 serotypes and some had as many as 6 serotypes. 3. Many of the relapse populations originating from 24 serotypes had organisms that were not stained homologously with any of the standard antisera. No attempt will be made to establish the actual number of serotypes that *Borrelia* can use to express its surface antigen, but is likely less than 50. At least, the number is far greater than the number of relapses experienced by man or laboratory animals. 4. After initial infection, the serotypes represented in the population in a given mouse usually persist for 2 days and, in most cases, the change from one serotype to another is gradual. However, in many instances, the serotype of the population in a mouse may change completely within 24 hours.

Future studies will be oriented toward elucidating the dynamics of seroconversion. It is contended by some that Borrelia constantly change their surface antigens and that the serotypes in an animal at a given time represent those to which the host has not produced antibodies. An alternative theory, not consistent with current concepts of genetics of microorganisms, holds that the antibody produced by the host stimulated the organism to change its surface antigens. It is known that Borrelia, in culture, in the absence of antibody, do not seroconvert. This finding suggests that the organism must interact with antibody before seroconversion occurs.

Publications:

Stoenner, H. G.: Q fever. In Steele, J. H. (Ed.): CRC Handbook Series in Zoonoses. Boca Raton, Florida, CRC Press, Inc., 1980, vol. II, pp. 337-352.

Stoenner, H. G.: Rickettsioses of domestic animals not transmissible to man. In Steele, J. H. (Ed.): CRC Handbook Series in Zoonoses. Boca Raton, Florida, CRC Press, Inc., 1980, vol. II, pp. 353-355.

Rocky Mountain Laboratory
Administrative and Operational Support
Operations Branch

1980 Annual Report
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Rocky Mountain Laboratories Operations Branch, NIH, NIAID
Administrative and Operational Support
October 1, 1979 - September 30, 1980

Introduction

The Branch provides all services necessary to the professional staff in the pursuit of their investigations. This support covers the following areas: procurement, personnel, custodial, security, media preparations, glassware refinishing, photography, animal rearing and care, motor pool, operation of power plants, and full maintenance in every area except electronics.

General Overview of the Responsibilities of Operations Branch

The fiscal and procurement department manages a budget of $1,275,000. Payroll is not included in this figure. It covers only the purchase of supplies and minor equipment used in the operation of the laboratories. Timekeeping and the correction of errors in the payroll are also handled in the unit.

Personnel handles all actions and advises on personnel matters. This department also is charged with the operation of the Comprehensive Employment Training Act (CETA) in association with the local Montana State Employment Office. Through the year we have averaged five (5) people on this program at all times. The maximum time a person may spend on the program is 3 months. Hence, we are constantly interviewing and employing people under the program. Also handled by Personnel are persons under the following programs: Stay in School, Work Study, Government Summer Program, and students studying for advanced degrees.

Custodial services are provided in the five laboratory buildings daily. Security is provided in the form of a guard on duty every night of the year.

Most of the media used in the laboratories is prepared at the lab under a contract by an outside contractor. All glassware used in the lab is cleaned and sterilized for reuse in the laboratories.

The photography department provides full professional services necessary in the laboratories with the exception of medical artistry.

The animal unit raises two strains of guinea pigs, five strains of mice, plus one strain of rats and hamsters, and one colony of microtus. They breed and raise approximately 145,000 animals a year. An additional 12,000 animals are purchased annually from outside sources, including mink, sheep, rabbits, mice, guinea pigs, and hamsters. After rearing, care is provided for these animals while they are under experiment.

The maintenance department, through the power plant, provides heat, steam, air and vacuum to the laboratories. Also provided are air conditioning, compressed air, vacuum and demineralized and distilled water. A motor pool consisting of 10 vehicles is maintained and grounds care, including snow removal, is provided.

With the exception of the electronics work, all maintenance is done by the staff. This includes plumbing, electrical, sheet metal, carpentry, and refrigeration, including ultra low temperature boxes.

Labor management work is handled by the Chief of the Branch.

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Bethesda, Md. 20205

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